The molecular genetics of Type 2 diabetes

Thesis

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THE MOLECULAR GENETICS OF TYPE 2 DIABETES.

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ABSTRACT.

THE MOLECULAR GENETICS OF TYPE 2 DIABETES.

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Type 2 diabetes has a substantial genetic component. The aim of this dissertation was to investigate the molecular basis of Type 2 diabetes with particular emphasis on the role of the glucokinase gene. Other work studied specific mutations in the $tRNA^{Leu(UUR)}$ gene of mitochondrial DNA and in the glucagon receptor gene.

The Introductory chapter presents an overview of Type 2 diabetes, and the influence of the environment and the contribution of genetics to its development. It then introduces the molecular biological approach to the study of this disease and the function of the β-cell and the important role of glucokinase in glucose homeostasis.

Chapter 2 gives details of ascertainment criteria for the subjects studied and outlines the methodology used to study the molecular genetics of Type 2 diabetes. The techniques described include DNA extraction, the polymerase

chain reaction (PCR), microsatellite markers, restriction fragment length polymorphisms (RFLPs), mutation screening, separation of PCR products and direct sequencing of them.

Chapter 3 investigates the role of the glucokinase gene in five pedigrees with maturity onset diabetes of the young (MODY). Using the microsatellite polymorphisms GCK1 and GCK2, diabetes was found not to be linked to the glucokinase gene in these multi-generation pedigrees.

Chapter 4 assesses the contribution of the glucokinase gene to Type 2 diabetes in the UK Caucasian population by investigating the two microsatellite polymorphisms in well-characterised Type 2 diabetic subjects and normoglycaemic control subjects. There was no linkage disequilibrium between the two polymorphisms, and no association with diabetes was found. This suggests that a single mutation in or near the glucokinase gene is not a common cause of Type 2 diabetes in this population.

Chapter 5 describes the development of a robust method to screen for mutations using the technique of single-stranded conformational polymorphism (SSCP) analysis. Chapter 6 utilises this method, and found that mutations in the glucokinase gene can contribute to the pathogenesis of gestational diabetes.

Four pedigrees from the Oxford district had been found to possess the same

missense mutation in the glucokinase gene at position 299. Chapter 7 establishes that the high prevalence of this glucokinase mutation in this district is probably due to a founder-effect, by haplotyping affected members of the four pedigrees for GCK1 and GCK2.

Chapters 8 and 9 use restriction fragment length polymorphism (RFLP) analysis to detect specific mutations known to create, or remove a cutting site for a restriction endonuclease. Chapter 8 investigates the contribution of the mitochondrial transfer RNA ($tRNA^{Leu(UUR)}$) mutation at position 3243bp to Type 2 diabetes. Chapter 9 studies a mutation in the glucagon receptor and its possible susceptibility to Type 2 diabetes.

Chapter 10 concludes the dissertation and outlines future areas for investigation.

CONTENTS

DECLARATION.

The molecular genetic studies presented in this thesis are my personal work, carried out under the supervision of Dr Robert Turner and Dr Andrew Hattersley. This included the use of microsatellite markers, restriction fragment length polymorphisms (RFLPs), and the setting up of single-stranded conformational polymorphism (SSCP) analysis and direct sequencing. The clinical characterisation of the diabetic families and gestational diabetic subjects reported in this thesis were performed by medical staff of the Diabetes Research Laboratories, Oxford. Dr Andrew Hattersley conducted the clinical investigations of 4 of the MODY pedigress and co-ordinated the follow-up of the gestational diabetic subjects. His practical work within this thesis includes the DNA extraction of the MODY pedigrees, and their molecular genetic analysis using the microsatellite marker GCK1. The statistical analysis for the population association study was performed by Dr Andrew Hattersley with Irene Stratton. The gestational diabetic subjects were initially identified by Mr Mike Gillmer. The blood samples and biochemical data on Type 2 diabetic patients and normoglycaemic controls were provided by the UK Prospective Diabetes Study (UKPDS). Subjects with fasting hyperglycaemia were from the Oxford centre of the Fasting Hyperglycaemia Study. Virginia Horton assisted with DNA extraction. The glucokinase gene expression studies and the computer assisted modelling of glucokinase were performed by Dr Gidh-Jain and his colleagues in the

Department of Physiology and Biophysics, State University of New York, New York. Dr Steve Gough set up the assay for the mutation in the glucagon receptor and screened the Type 2 diabetic subjects, whereas I screened the subjects with gestational diabetes, fasting hyperglycaemia, the MODY and nuclear pedigrees, and normal control subjects. The statistical analysis in this thesis was performed with the assistance of Irene Stratton, and Dr Andrew Hattersley.

CHAPTER 1

INTRODUCTION

1.1 TYPE 2 DIABETES.

Type 2 diabetes is a major cause of morbidity and mortality in both the developed and developing world. Morbidity is associated with microvascular, macrovascular and neurological diseases, and cardiovascular mortality increase is 2-3 fold. The prevalence of the disease varies. In Caucasian populations it ranges between 2-6%, but in some ethnic groups exceeds 30% (Zimmet 1982). An increase in the understanding and pathophysiology of this important disease will allow developments in its treatment, and possible prevention (UKPDS VIII).

Diabetes mellitus is differentiated into two groups; Type 1 diabetes which is due to autoimmune destruction of the insulin secreting pancreatic β-cells. Type 2 diabetes is not an autoimmune disease, subjects are islet cell antibody negative and their hyperglycaemia may initially be controlled with diet and/or tablets, but may progress to requiring insulin treatment although they are not insulin-dependent. Differentiation between the two forms of diabetes has been aided by HLA typing and the identification of specific alleles being associated with Type 1 diabetes (Cudworth et al 1974; Nerup et al 1974). Problems with diagnosis of Type 2 diabetes occur as there is no biochemical marker for the disease and treatment decisions, particularly with regard to insulin use, are physician dependent. Subjects tend to present in middle or old age (age 50-60 years), unless they are screened in pregnancy or present with another illness. A large number of cases are subclinical and so complications may occur before the

diagnosis of diabetes is made.

Type 2 diabetes arises due to both β-cell defects and insulin resistance resulting in inappropriate insulin secretion for the levels of circulating glucose (DeFronzo et al 1988; Eriksson et al 1989). Both of these contribute to Type 2 diabetes, along with obesity, and our present knowledge does not enable us to determine that any one is the "primary defect" of Type 2 diabetes. Nevertheless, there are some subgroups of the disease which are due to only one defect. For example, patients with Maturity Onset Diabetes of the Young (MODY) only have a β-cell defect, and do not have increased insulin resistance compared to non-diabetic subjects (Hattersley et al 1992b).

1.2 ENVIRONMENT.

The environment is an important factor in the development of the disease. Improved understanding of its influences will help to define the genetic factors with which they interact.

1.2.1 High risk populations.

The Pima Indians of Arizona and the Nauruans of Micronesia are two isolated populations in whom Type 2 diabetes was previously unknown. Over the last 50 years it has now reached almost epidemic proportions and has a prevalence of

over 30% in adult Pima Indians and Nauruans (Zimmet et al 1981). This increase has been associated with dramatic changes in lifestyle. Both populations had traditionally been active hunter-gatherers, but due to westernisation have now become sedentary, and many are now greatly obese. Studies comparing rural to urban migration related differences in prevalence have shown the importance of these lifestyle changes (Zimmet et al 1983; Taylor et al 1985; Serjeantson et al 1983). There is a higher prevalence of diabetes in subjects living in a modern urbanised environment, than those who maintain a relatively traditional lifestyle. An urban environment does not result in such a high prevalence in all populations, so there must be a difference in susceptibility (Zimmet et al 1983). This difference in susceptibility is probably genetic; in high prevalence populations the frequency of diabetes declines as the proportion of foreign genes increases. In full-blood Nauruans the prevalence of diabetes after the age of 60 years is 83%, whereas it is 17% in those inhabitants in whom Caucasian ancestral gene admixture, assessed by HLA typing, can be demonstrated (Serjeantson et al 1983).

JV Neel proposed the "thrifty genotype" to explain the increase in prevalence with westernisation. The same gene or genes which gave selective advantage during a hunter-gatherer existence where feasting was followed by long periods of starvation, are now responsible for the high prevalence of diabetes in an environment which now has an abundant supply of food (Neel JV 1962). The "thrifty genotype" would favour a highly efficient storage of energy as fat and

glycogen which would be detrimental when there is a constant supply of refined carbohydrate. The precise pathophysiology of the "thrifty genotype" is poorly defined but offers an interesting theory with regards to the relationship between inherited and environmental factors in these high prevalence populations.

1.2.2 Age, Obesity and Physical Activity.

The incidence of diabetes increases with age in Caucasian populations. This could either represent a physiological increase in all population members' glucose tolerance or an age-related expression in those members with a genetic predisposition. The increase in insulin resistance associated with obesity, possibly due in part to the increased supply of non-esterified fatty acids, will increase the hyperglycaemia in a subject with Type 2 diabetes. Exercise will increase insulin sensitivity and physically fit people have a lower incidence of diabetes. It is possible that obesity and a sedentary lifestyle are major precipitators of the expression of at-risk genes for diabetes.

1.2.3 Intrauterine environment.

Hales et al observed that a low birth weight was associated with glucose intolerance in old age in a cohort of men from Hertfordshire. This led to the suggestion that intra-uterine environment may be important in the aetiology of Type 2 diabetes (Hales et al 1991). It has been proposed that intra-uterine

nutrition may affect the development of the foetal pancreas and so alter the adult pancreatic reserve, or it may affect insulin resistance. An alternative explanation is that the genetic predisposition to diabetes results in reduced foetal size as well as the lower birth weight.

1.2.4 Viral infection.

Viral infections may have an important role in the progression of Type 1 diabetes. Cytomegalovirus (CMV) has been seen with greater frequency in pancreatic samples from Type 2 diabetic subjects than pancreatic samples from controls (Lohr and Oldstone 1990). It has been proposed that low level chronic infection of β-cells could be responsible for the relative hyposecretion of insulin seen in Type 2 diabetic subjects. Hattersley et al failed to replicate the observations and suspected that contamination of pancreatic sections had occurred in the original study (Hattersley et al 1992a). Viral infection still remains a possible contributor to the pathogenesis of Type 2 diabetes, but this remains to be substantiated.

1.3 GENETICS OF TYPE 2 DIABETES.

1.3.1 Early Studies.

Genetic factors contribute to the aetiology of Type 2 diabetes (Kobberling and Tillil 1982). Early clinical descriptions reveal that the familial nature of the

disease was not recognised. This was probably due to the rarity of the disease at the time (Galen only observed two cases during his career), and that most cases were likely to be Type 1 diabetes in whom family members are less likely to be affected. Sushruta and Charuka in the 5th century AD were probably the first to record the familial nature of diabetes (Simpson 1976). In 1696, Richard Morton described a pedigree in which 4 out of 7 siblings had diabetes mellitus. He concluded that this occurred due to the inheritance of a common familial factor predisposing to diabetes.

Early in the 20th century, it was recognised that a family history of diabetes was more common in diabetic patients than non-diabetic patients. Pincus and White found a family history in 23% of close relatives of a cohort of 523 diabetic patients, as opposed to just 10% in comparable relatives of 153 controls (Pincus and White 1933). Cummidge recognised that there were two forms of diabetes which probably had two different modes of inheritance (Cummidge et al 1928). One form showed dominant inheritance which "is invariably mild and may persist for many years", or recessive which "is generally grave from onset". The significance of this was not fully appreciated, and studies did not differentiate/distinguish between the two forms of diabetes. Differentiating Type 1 and 2 diabetes was vital in reducing heterogeneity and to clarify the inheritance of diabetes. Further observations were reported which were important in separating the two forms of diabetes.

Harris reported on the family history of a series of 1241 patients from King's College Hospital clinics (Harris 1950). He discovered that when two siblings had diabetes there was a marked similarity in their age of onset. Siblings of a subject with an earlier age of onset were more likely to develop diabetes earlier in their life than siblings of late onset subjects. He concluded that there was genetic heterogeneity between early and late onset diabetes.

1.3.2 Studies in Twins.

Twin studies have provided strong evidence for a major genetic component in Type 2 diabetes. Early twin studies had revealed a higher concordance rate (45-96%) in monozygotic (identical) twins than dizygotic (non-identical) twins (3-37%) (reviewed in Barnett et al 1982). The authors did not discriminate between Type 1 and 2 diabetes, or perform glucose tolerance testing in the "unaffected" twin, which makes interpreting these results difficult. The concordance rate would have been underestimated, as Type 2 diabetes is often sub-clinical.

Barnett reported on the King's longitudinal collection of 200 pairs of monozygotic twins who had been ascertained by one twin already having diabetes (Barnett et al 1981a). The subjects were sub-divided into Type 1 and 2 diabetes, and regular testing of glucose tolerance was carried out. 48/53 (91%) of Type 2 diabetic twins were concordant, in contrast to 80/147 (54%) of Type 1 twins (Barnett et al 1981a). In 46/48 concordant Type 2 diabetic twin pairs, the second twin was

diagnosed as having diabetes within 10 years of the first. The 5 individuals who had been shown to be discordant for over 15 years were shown to have mild hyperglycaemia and low insulin responses to an oral glucose load (Barnett et al 1981b). These results could reflect early alterations leading to the development of Type 2 diabetes, which would suggest that concordance is nearing 100%. The authors were aware of selection bias in their study which would favour finding concordance. Those cases with both twins affected were more likely to be referred to this study than cases where only one twin was affected.

Newman avoided ascertainment bias by identifying 250 monozygotic and 264 dizygotic twins through military records, without reference to disease status (Newman et al 1987). Only 4/25 twins were concordant for Type 2 diabetes when they were first examined at a mean age of 47 years. 10 years later, only 1/15 of the originally discordant monozygotic twin pairs retested remained discordant. These findings show that if an unaffected monozygotic twin of a Type 2 diabetic patient is followed up with glucose tolerance testing for 10 years, there is a high probability that they will be diagnosed with diabetes too.

This data is regarded as strong evidence for a genetic contribution to Type 2 diabetes. The high concordance in Type 2 diabetes could be due to a shared genetic predisposition, or a shared environment. Evidence to support that the genetic factors are more important comes from the lower concordance rates seen in dizygotic twins (Newman et al 1987), and that twins live apart in their middle

and latter lives when Type 2 diabetes is diagnosed (Barnett et al 1981a; Newman et al 1987). The delay in the diagnosis of the second twin suggests that environmental factors influence the timing of the onset of diabetes.

1.4 THE GENETIC NIGHTMARE OF TYPE 2 DIABETES.

Despite the fact that Type 2 diabetes is a common disease and has been shown to have a large genetic component, it has been difficult to identify the genetic defects involved in its aetiology. Type 2 diabetes has been described as the "genetic nightmare" (Neel 1976; O'Rahilly et al 1988). The study of the molecular biology of Type 2 diabetes has been hindered by the heterogeneity of the disease, and difficulties in defining affection status, collection of large multi-generation pedigrees, and defining the pattern of inheritance. Segregation analysis has shown that Type 2 diabetes is unlikely to be due to a recessive gene, or a single autosomal dominant gene with high penetrance (Cook et al 1993), although this may be the case in a minority of pedigrees. It appears to be polygenic, although a major gene may be present (Cook et al 1993).

1.4.1 Collection of large, multi-generation pedigrees.

The technique of linkage analysis is a very powerful tool defining the molecular biology of diseases which is best applied to large multi-generation pedigrees with a single gene disease (Ott 1985). Collection of large multi-generation pedigrees

is hampered by the late age of onset of the disease and the disease-related mortality. Type 2 diabetes usually presents in the 6th decade. Children of affected individuals will be in their 30s and 40s at which time it is impossible to define if they will go on to develop diabetes in later life. The parents of the affected individuals are likely to be in their 70s and 80s and frequently have died. The disease-related mortality will mean that the parents are more likely to have died if they had suffered from diabetes, whether it had been diagnosed or not. There is an approximately two fold increase in mortality in subjects with diabetes generally as a result of cardio-vascular deaths (Panzram et al 1987). Probands diagnosed before the age of 40 years increases the likelihood that both parents will be alive, but the early age of diagnosis of the proband is frequently associated with bi-lineal inheritance. O'Rahilly found that in subjects with "early-onset diabetes" (diagnosed between the ages of 25 and 40 years), 83% of subjects had both parents affected with either diabetes or glucose intolerance (O'Rahilly et al 1987). Linkage analysis gives very little information, if any, when both of the parents are affected.

1.4.2 Heterogeneity.

To overcome the problems of collecting large multi-generation pedigrees, an alternative is to collect many small nuclear families. This strategy is likely to be unsuccessful in Type 2 diabetes as it is heterogeneous, rather then homogeneous. The heterogeneity of Type 2 diabetes is already apparent as

different genetic causes may have a similar diabetic phenotype including haemochromatosis, Turner's syndrome, Prader Willi syndrome and cystic fibrosis (Vadheim and Rotter 1992). In rare cases, hyperglycaemia can also occur because of mutations of insulin (Steiner et al 1990; Taylor et al 1993), or the insulin receptor as in leprechaun syndrome. Within the Type 2 diabetic population there is considerable variation in obesity, degree of hyperglycaemia, and pathophysiology. Phenotypic variation suggests genetic heterogeneity, but this will not be clarified until genetic markers are defined. To reduce the problems of heterogeneity specific, well-defined populations may be studied. Examples of such subgroups are those with insulin resistance, or maturity onset diabetes of the young (MODY).

MODY has an early age of onset (before the age of 25 years in at least two family members), not insulin-dependent (shown by absence of insulin treatment 5 years after diagnosis or significant C peptide in a patient on insulin treatment), and autosomal dominant inheritance (ie vertical transmission of diabetes through at least three generations, ideally with the same phenotype in cousins or second cousins) (Hattersley and Tattersall 1995). Physiological studies have shown that MODY is a β-cell defect (Tattersall 1975; Fajans 1989; Hattersley et al 1992c). Studying the MODY subgroup has the advantages of enabling collection of suitable large multi-generation pedigrees, and reducing the amount of genetic heterogeneity.

1.4.3 Difficulties in defining the affection status.

To investigate the molecular genetics of a disease such as diabetes, well-characterised affected and non-affected subjects and collection of large, multi-generation pedigrees are required for the studies. A particular problem encountered with diabetes is that it may be sub-clinical and hence undiagnosed. This may alter the conclusions of the genetic study. Therefore, physiological studies should accompany genetic studies to aid the biochemical assessment of subjects and defining of study populations.

1.5 MOLECULAR BIOLOGICAL APPROACH.

The main approach in the investigation of the molecular genetics of Type 2 diabetes has been the study of candidate genes. Localisation of a candidate gene to a particular chromosome through the identification of its cDNA sequence enables study of its contribution to the disease.

1.5.1 Molecular biological markers.

Molecular genetic studies of Type 2 diabetes have principally used population studies and linkage studies. These approaches both utilise molecular markers which are shown to be linked to the gene of interest. Restriction fragment length polymorphisms (RFLPs), or more recently polymorphic microsatellites which are

both located throughout the human genome are used as genetic markers.

RFLPs are sites within the genome which are recognised by specific restriction endonucleases, these enzymes will then cut the genomic sequence. The RFLPs are detected as differences in the size of fragments produced after enzyme digestion which can be resolved by electrophoresing of DNA on polyacrylamide gels followed by Southern blotting, or by PCR amplification of a specific restriction site and running the digested PCR product on agarose gels and ethidium bromide staining.

Microsatellites are di-, tri-, or tetra- nucleotide repeats that show length polymorphisms resulting in the variation in the number of repeats. Following polymerase chain reaction (PCR) amplification of these microsatellite sequences, the microsatellite polymorphisms (alleles) are resolved on agarose gels and their sizes determined. These microsatellite polymorphisms may be used as markers if they are so close as to exclude recombination between themselves and the candidate gene. The length polymorphisms (alleles) can then be studied in pedigrees and populations, and then assessment made as to whether the candidate gene increases susceptibility to disease.

1.5.2 Linkage analysis.

Linkage analysis is performed on suitable multi-generation pedigrees to study the

co-inheritance of molecular markers with disease. When the marker is in close proximity to the candidate gene they will be inherited together. If a particular marker allele is found to cosegregate with the disease there could be a defect in the candidate gene that is inherited with it. Statistical calculations (LOD scores) are carried out to assess whether there is significant linkage of the candidate gene to the disease. By convention, a positive LOD score of +3 or greater is considered as evidence of linkage, and a LOD score of less than -2 is taken as evidence of exclusion of linkage for the particular recombination fraction (θ) (Lathrop and Laloud 1984).

1.5.3 Population association studies.

Population association studies have an important role in determining the genetic basis of a disease. Polymorphic molecular markers are used and investigated in non-affected (normal) and affected subjects. The frequency of marker alleles or haplotypes (linkage of two or more polymorphic sites along a chromosome) are compared between the two groups. The frequency of a marker allele will be increased in the affected group if that allele is in linkage disequilibrium with a common mutation in the candidate gene. Linkage disequilibrium implies that a specific disease-causing mutation originally arose in one individual or a small population of subjects and that they possessed a specific allele at a nearby genetic marker locus. The two loci are physically so close that throughout the subsequent generations of descendants these loci have not been separated

during meiotic recombinations. The marker may then be used to study the association of a particular allele with disease in a population.There are problems associated with this type of investigation:

a. The genetic marker must be tightly linked to the candidate gene.

b. There must be one or only a few defects in the candidate gene causing the disease, otherwise the mutations will be found randomly with different alleles of the marker loci.

c. The mutations must have a recent origin, otherwise the mutations will be randomly associated with different alleles of the marker loci.

d. Affected and non-affected subjects must be from the same ethnic population (see Genetic Admixture).

e. The information acquired is highly dependent on the degree of polymorphisms of the marker loci studied.

1.5.4 Genetic Admixture.

Genetic admixture is a complicating factor in population association studies. A population which appears to be homogeneous may in fact have genetic

components from an ethnic group. Any trait at a higher frequency in an ethnic group will show positive association with any allele that also happens to be common in that group. An example of foreign genetic admixture is seen in Nauruans. There is a higher frequency of Type 2 diabetes in full-blooded Nauruans compared to those with Caucasian ancestral gene admixture, as assessed by HLA typing. Therefore, any polymorphism that is of a higher frequency in Nauruans compared to Caucasians will be associated with diabetes. To reduce the risk of obtaining an incorrect association, the following steps may be taken:

a. they should be performed in a homogeneous population.

b. use an "internal control" for allele frequencies. This studies the inheritance of alleles in affected individuals and their parents (transmission distribution of kin test)

c. perform a transmission disequilibrium test (TDT). This assumes that a parent heterozygous for an associated allele (A) and a non-associated allele (a), should transmit (A) more often than (a) to an affected subject.

1.5.5 Mutation screening.

Sequencing is the most accurate method of identifying mutations in candidate

genes, but this is time-consuming, expensive and laborious. Rapid methods of screening subjects to detect those which may possess a mutation can be employed prior to sequencing. The most frequently used method is to screen for single-stranded conformational polymorphisms (SSCP) (Orita et al 1989). A region of DNA is amplified (usually exons) using PCR, the product is heated to denature it to single strands, and then run on a non-denaturing gel. The base sequence of the single-stranded DNA will determine the secondary structure, this will affect its mobility on the gel. Mutations are detected as differences in mobility of the sample on the gel. SSCP is rapid and allows the detection of mutations even in the presence of heterogeneity. It is unlikely to detect all mutations but under optimal controlled conditions it is reported to have a sensitivity of between 68 - 99% (Orita et al 1989; Sheffield et al 1993). Once an SSCP variant is found sequencing of the DNA is undertaken to identify the base change responsible which may help to determine whether it is a polymorphism or mutation. A silent polymorphism is clearly unlikely to be the cause of disease. Sequence variations which alter a coding region may still not be the causative mutation. The contribution of the mutation to the disease will still need to be determined.

1.5.5 Biological significance of a mutation.

Silent polymorphisms are unlikely to be the cause of a disease. Some sequence variations such as frame shifts, or chain terminations, are obviously deleterious to a protein. Other alterations such as missense mutations leading to amino acid

substitutions, or base changes within introns, can be either functionally relevant or sequence polymorphisms. To determine whether a sequence alteration is pathogenic, family studies, population studies and functional assays after in vitro expression may be required.

Many candidate genes and their role in diabetes mellitus have been considered. The main part of my thesis concentrates on the contribution of defects in the glucokinase gene to Type 2 diabetes, with additional data on mitochondrial DNA and the glucagon-receptor gene. These are reviewed in the following sections.

1.6 THE β-CELL AND THE ROLE OF GLUCOKINASE IN INSULIN SECRETION.

Glucose homeostasis is regulated by insulin; the secretion of insulin from the pancreatic β-cell is itself regulated by plasma glucose concentrations within a negative feedback mechanism. In a normal subject, insulin is secreted appropriate to the levels of circulating glucose. This prevents wild fluctuations in the levels of glucose and the effects of complications from hypo and hyperglycaemia.

Glucokinase (GCK) is an important enzyme in the regulation of glucose metabolism in the pancreatic islets and liver, and has been described as the "pancreatic glucose sensor" (Matschinsky 1990). Glucokinase catalyses the phosphorylation of glucose to glucose-6 phosphate, the first step in the glycolytic

pathway.

Glucokinase (ATP: D-hexose-6-phosphotransferase; EC 2.7.1.1.) is a member (hexokinase IV) of a family of hexokinases (I-IV). It is distinguishable from the other hexokinases by its lower molecular weight, higher Km for glucose (5mM) which is in the physiological range (4-15 mM), sigmoid glucose - reaction velocity relationship (Hill coeffecient 1.6) and by its lack of product inhibition by glucose-6-phosphate. These unique kinetic properties result in glucose phosphorylation maintaining a gradient for glucose transport, and allows the β-cell and hepatocytes to respond appropriately to the degree of hyperglycaemia (Matschinsky 1990; Meglasson and Matschinsky 1986).

Glucose is passively transported into the pancreatic β-cell down its concentration gradient by the liver/islet glucose transporter (Glut 2). Glucokinase then catalyses the phosphorylation of glucose, producing glucose-6-phosphate. This product is metabolised by glycolysis and then progresses from the cytoplasm to the mitochondria where oxidative phosphorylation occurs. This results in the elevation of the ratio of ATP/ADP concentrations, catalysing the closure of the ATP-dependent potassium channels in the cell membrane (Ashcroft and Ashcroft 1992). Depolarisation of the cell membrane causes the opening of the voltage-dependent calcium channels. The intracellular calcium levels increase which leads to exocytosis of insulin granules (Figure 1.6). Glucose concentration is the major regulator of glucokinase expression within the β-cell (Magnuson 1990).

Hyperglycaemia will increase expression of glucokinase and hence its activity, this then results in an increase in insulin secretion.

The primary response of the liver to hyperglycaemia is an increase in the uptake of glucose and the synthesis of glycogen. This is partly regulated by glucokinase (Matschinsky 1990). Glucose phosphorylation maintains a concentration gradient for glucose transportation across the cell membrane via Glut 2. This enables glucose uptake by the liver and its metabolism to glycogen. Expression of glucokinase within the liver is increased in response to an elevation in insulin levels (Magnuson 1990), which results in an increase in hepatic glucose uptake following meals.

Glucokinase is a good candidate gene for Type 2 diabetes. It is only expressed in pancreatic β-cells and liver hepatocytes, this is tightly controlled by two tissue-specific promoters. There are 3 glucokinase mRNA isoforms (1 for β-cells & 2 for liver) which are formed by the differential splicing of mRNA. Mutations in this gene which could cause alterations in the structure and function of the enzyme it codes for may have a profound effect on its capability to act as the "pancreatic glucose sensor". Defects in the glucokinase enzyme could reduce the ability of the pancreatic β-cell islets and liver hepatocytes to respond to fluctuations in plasma glucose and maintain the body's glucose homeostasis.

1.7 THE CONTRIBUTION OF THE GLUCOKINASE GENE TO TYPE 2 DIABETES.

The cDNA sequence of glucokinase in the rat was identified in 1989 (Matschinsky et al 1989). It was found that there were tissue-specific isoforms which differed in exon 1 (Magnuson and Shelton 1989; Magnuson et al 1989). This meant that differential expression in the pancreatic β-cells and liver hepatocytes was possible, regulated by glucose. These findings brought the possibility of molecular biological and genetic studies to determine whether defects in the glucokinase gene had a role in Type 2 diabetes. A microsatellite polymorphism in the form a of a dinucleotide repeat $(CA)_n$ tightly linked to the glucokinase gene was identified (Matsutani et al 1992). This enabled linkage studies and population association studies to be carried out.

Tight linkage of the glucokinase gene with Type 2 diabetes was found in pedigrees with maturity onset diabetes of the young (Froguel et al 1992; Hattersley et al 1992b). Mutations of the glucokinase gene were subsequently identified in affected subjects (Vionnet et al 1992; Stoffel et al 1992b). A missense mutation in exon 8, resulting in the substitution of $Gly^{299} \rightarrow Arg$, was found in Pedigree BX. This same mutation was found in subjects from a classical Type 2 diabetic family (Pedigree AX), after a member was picked up on screening of 100 English Caucasian subjects with Type 2 diabetes. Screening of 100 unrelated non-diabetic subjects failed to find this mutation, thus strengthening the

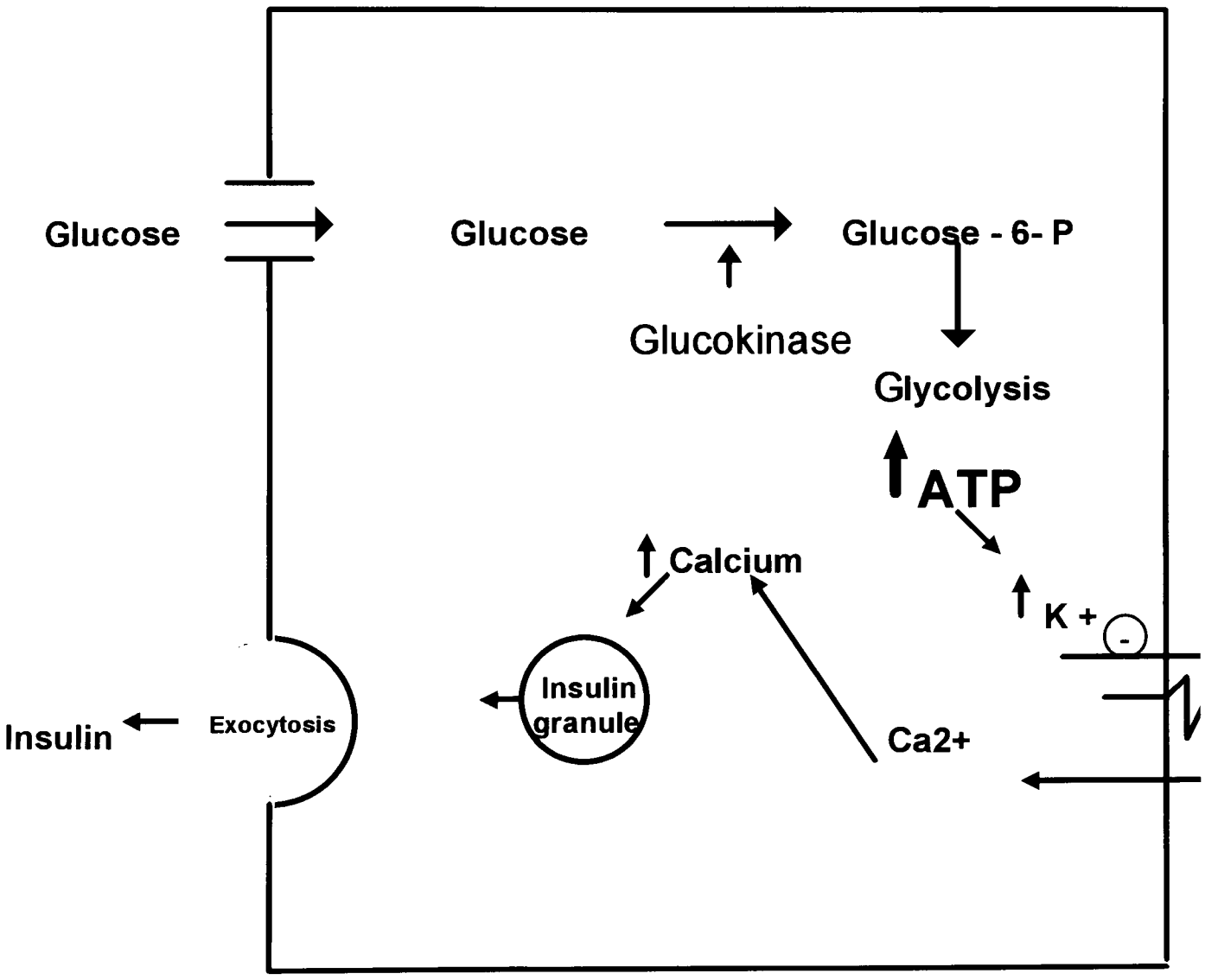
conclusion that this mutation was the cause of diabetes (Stoffel et al 1992b).

Modelling of human glucokinase, using the related yeast hexokinase, has shown that exon 8 codes for a region of the enzyme which leads to the cleft of the active site where glucose is bound to enable the catalysation of phosphorylation (Gidh-Jain et al 1992; StCharles et al 1994). Expression studies in E.coli have shown that this missense mutation in exon 8 reduces the affinity (Km) of the enzyme for glucose to less than 1% of that for the native form (Gidh-Jain et al 1992).

Glucokinase mutations have been found in subjects in a Japanese pedigree with early-onset Type 2 diabetes (Sakura et al 1992) and with late-onset Type 2 diabetes (Katagiri et al 1992). The phenotypes of these subjects is similar to that seen in the British and French MODY pedigrees. The difference in the age of diagnosis and hence classification is probably due to later glucose tolerance testing rather than a different phenotype.

Positive population association studies with GCK1 in Mauritian Creoles and Black Americans (Chiu et al 1992a & b) have been published; these suggest a role for defects in the Glucokinase gene in the development of Type 2 diabetes. Another polymorphism in tight linkage to the GCK gene has been found (GCK 2) (Nishi et al 1992) which may be used as a marker for the gene. When used in tandem with GCK 1, haplotypes may be constructed when a subject is homozygous at one or more loci. This increases the power of population association studies and allows

Figure 1.6 Diagram of β-cell showing the role of glucokinase.



This schematic diagram shows the pathway linking glucose with insulin release in the pancreatic ß-cell showing the central role of glucokinase.

linkage disequilibrium between the two markers to be assessed.

1.8 MITOCHONDRIAL DNA AND THE $tRNA^{Leu(UUR)}$ MUTATION AT POSITION 3243BP.

Mitochondria are organelles within cells which contain their own DNA. Each cell contains thousands of mitochondria and each mitochondrion contains 2-10 copies of mitochondrial DNA (mtDNA). One theory of evolution suggests that mitochondria were previously separate, they were engulfed by cells and embarked on a symbiotic relationship. Human mtDNA is circular, consisting of 16,595 bp and has been fully sequenced (Anderson et al 1981). It encodes 13 subunits of the respiratory chain and oxidative phosphorylation enzymes, 22 transfer RNAs (tRNAs) and 2 ribosomal RNAs (rRNAs). mtDNA is exclusively maternally inherited, probably because sperm contribute almost no cytoplasm to the zygote, as the tail is lost at fertilisation (Giles et al 1980).

The mitochondrial $tRNA^{Leu(UUR)}$ mutation at position 3243 bp, resulting in a G→A substitution, has been shown to occur within pedigrees with myoencephalopathy with lactic acidosis and stroke-like episodes (MELAS), and also in some subjects with chronic progressive external opthalmoplegia (CPEO) and in Kearns-Sayre syndrome (KSS) (Goto et al 1990). Subsequently, this same mutation was found to be present in a pedigree whose affected members had diabetes and/or deafness with a maternal mode of inheritance (van den Ouweland 1992. Other

similar pedigrees have since been reported (Reardon et al 1992; Kadowaki et al 1993; Remes et al 1993; Alcolado et al 1994; Katagiri et al 1994). The differences in the diseases and the clinical phenotypes shown may be due to several factors, including the degree of heteroplasmy. Normal and mutant mtDNA are present in the same tissue, and the proportion of mutant mtDNA varies between tissues and individuals. There may also be an effect with nuclear-encoded mitochondrial enzymes.

Mutations in mitochondrial DNA are thought to affect glucose homeostasis. Under aerobic conditions, 95% of ATP synthesis in β-cells is supplied by the mitchondrial respiratory chain. An increase in the ATP/ADP ratio within the β-cell results in closure of the ATP-dependent potassium channels in the cell membrane. Depolarisation of the cell membrane opens the voltage-dependent calcium channels. The intracellular calcium levels increase which leads to exocytosis of insulin granules. Defects in mitochondria may affect this process and lead to diabetes mellitus. The prevalence of the $tRNA^{Leu(UUR)}$ mutation at position 3243 bp within the UK Type 2 diabetic population needs to be assessed.

1.9 A HETEROZYGOUS POINT MUTATION IN THE GLUCAGON RECEPTOR GENE.

Glucagon has an important role in the regulation of hepatic glucose production and also has a minor role in the regulation of insulin secretion. Glucagon action

is mediated via its binding to a specific receptor belonging to the superfamily of G protein-coupled transmembrane receptors. Mutations in the glucagon receptor gene located on chromosome 17q25 may therefore contribute to Type 2 diabetes.

A point mutation in exon 2 ($Gly^{40} \rightarrow Ser$) has been found to be associated with Type 2 diabetes in French familial subjects (Hager et al 1995). Receptor binding studies found that this mutation results in a 67% reduction in the affinity for the binding of glucagon compared to the "wild-type". The physiological model is difficult to interpret, but this mutation may play a role in the development of Type 2 diabetes. This association study needs to be replicated in an independent cohort, and further studies are required to clarify the contribution of this mutation to Type 2 diabetes.

1.10 AIMS OF THIS THESIS.

The major questions that the work in this thesis set out to answer were:

1. To establish whether diabetes was linked to the glucokinase gene in five MODY pedigrees.

2. To assess the contribution of the glucokinase gene to Type 2 diabetes by performing a population association.

3. Development of single-stranded conformational polymorphism analysis, and consequently to assess the contribution of glucokinase mutations to gestational diabetes.

4. To investigate the prevalence of the previously described mitochondrial transfer mutation at position 3243bp, and assess its contribution to Type 2 diabetes, MODY and gestational diabetes in UK Caucasian subjects.

5. To investigate the prevalence of the previously described mutation in the glucagon-receptor and assess its role in Type 2 diabetes.

CHAPTER 2

METHODS

2.1 SUBJECTS STUDIED.

2.1.1 Introduction.

The subjects studied in the investigations in this dissertation were members of Type 2 diabetic and MODY pedigrees, random Type 2 diabetic subjects, gestational diabetic subjects, fasting hyperglycaemic subjects, and normoglycaemic subjects. This section outlines the selection criteria for these groups.

2.1.2 Pedigree selection criteria.

Pedigree members were considered affected if they:

1 had been diagnosed as having diabetes by WHO criteria (fpg >7.8mmol/l, or 2 hour post glucose load plasma glucose of >11.2mmol/l), and were on therapy with oral hypoglycaemic agents, insulin or as a specific diet.

2 had a fasting plasma glucose concentration >6 mmol/l, which is >3 standard deviations above the mean of a normal population.

3 had a plasma glucose >9.3 mmol/l, one hour after a continuous infusion of glucose 5mg/kg ideal weight per minute.

Pedigree members were considered unaffected if they had a fasting plasma glucose <6mmol/l or had a plasma glucose <9.3 mmol/l, one hour after a continuous infusion of glucose 5 mg/kg ideal weight per minute. As this disease has a late age of onset the diagnostic certainty of a normal test increases with age and this was taken into account in the computation of penetrances for linkage analysis.

2.1.3 Additional criteria for defining MODY pedigrees.

1 age at diagnosis of at least two affected members of the family was <25 years.

2 diabetes could be treated for at least 2 years without insulin.

3 the occurrence of diabetes in a family was suggestive of an autosomal dominant mode of inheritance (at least three generations of diabetes, and/or second cousins with Type 2 diabetes before the age of 25 years).

2.1.4 Ascertainment of pedigrees.

3 large pedigrees with MODY (BX, H, A) and a large pedigree with Type 2 diabetes (AX) were ascertained as part of a large UK study based from Oxford. All participating members gave informed consent. Probands were obtained

through a diabetic clinic and all relatives known to have diabetes were contacted and asked to provide a blood sample for fasting plasma glucose and DNA extraction. Their medical records were examined where possible. All other pedigree members were tested with either a fasting plasma glucose or a continuous infusion of glucose test. The majority of samples were collected by Dr AT Hattersley.

The MODY pedigree M was obtained through Dr Peter Watkins, King's College Hospital, and is one of the three original MODY pedigrees described by Professor Robert Tattersall.

The MODY pedigree EA has members residing in Edinburgh and Australia and was recruited by Dr MacKie and Dr J Baird. Samples from the Australian branch of the pedigree were obtained by Dr RC Turner.

Pedigree **N** was obtained from Cambridgeshire through Dr S O'Rahilly.

2.1.5 Random Type 2 diabetic subjects.

Blood samples were obtained from unrelated Caucasian Type 2 diabetic subjects from the UK Prospective Diabetes Study cohort and DNA was extracted. The subjects had newly diagnosed diabetes, age range 25-65 years inclusive (mean age 52 years),with fasting plasma glucose >6mmol/l on two separate occasions

mean 11.4mmol/l (UKPDS VIII, 1991).

2.1.6 Gestational diabetic subjects.

50 unrelated Caucasian subjects from the Oxford region who had Gestational diabetes were studied. Gestational diabetes was diagnosed on the basis of 2 abnormal oral glucose tolerance test (OGTT) values during pregnancy (28-34 weeks) by Mr Mike Gillmer, and with hyperglycaemia (>5.5 mmol/l <10 mmol/l) on follow up (mean 10 years). Subjects known to be members of pedigrees AX and BX were excluded from the study.

2.1.7 Fasting hyperglycaemic subjects.

DNA was extracted from blood samples from 100 subjects registered with the Oxford clinic of the Fasting Hyperglycaemia Study. Their fasting plasma glucose concentration was >5.5mmol/l and <7.8 mmol/l on two separate occasions.

2.1.8 Normoglycaemic control subjects.

DNA was extracted from blood samples obtained from 150 healthy Caucasian individuals recruited via the UKPDS. These subjects had no family history of diabetes and a fasting plasma glucose concentration <5.5 mmol/l.

2.2 PREPARATION OF GENOMIC DNA FROM PERIPHERAL WHOLE BLOOD.

1. Peripheral whole blood samples were collected in tubes containing either Sodium Heparin, or EDTA as anti-coagulant.

2. The samples were spun in a MSE refrigerated centrifuge at 1500g for 20 minutes at 4°C.

3. The plasma was then removed using a disposable pipette and discarded. The remaining cells were either stored at -20°C for processing later, or processed immediately.

4. The cells from the sample were transferred to a 50ml centrifuge tube.

5. To lyse the red cells 45ml of distilled water was added.

6. The tube was capped and whirlimixed, then centrifuged at 1500g for 15 minutes at 4°C.

7. The supernatant was carefully poured away.

8. 10 ml of 0.1% NP40 solution was added to the nuclear pellet.

9. Steps 6 and 7 were repeated.

10. 10 ml of lysis buffer was added to the pellet and then capped and whirlimixed.

11. 100µl of 10% SDS and 100mg of Proteinase K (1mg/ml) were added.

12. The tube was capped and the contents mixed gently by inverting, then incubated at 37°C for a period of 4 - 6 hours.

13. 2ml of 5x ANE was added.

14. 0.5 volumes of Tris- saturated phenol and 0.5 volumes of chloroform in amyl alcohol (CIAA) were added.

15. The tube was capped and the contents mixed thoroughly by inversion, then centrifuged at 1500g for 15 minutes at 4°C.

16. The aqueous layer was transferred into a clean 50ml centrifuge tube using a glass pipette.

17. Steps 14, 15 and 16 were repeated.

18. 1.0 volumes of CIAA was added to remove any contaminating phenol.

19. Steps 15 and 16 were repeated.

20. The DNA was precipitated by adding 0.1 volumes of 4M NaCl and 2.0 volumes of ethanol.

21. The tube was then capped and the contents mixed thoroughly by inversion, it was then incubated at -20°C for approximately 30 minutes.

22. The DNA was pelleted by centrifuging at 1500g for a minimum of 30 minutes at 4°C (the length of centrifugation will affect the yield).

23. The supernatant was gently poured off and discarded. The tube was inverted and the pellet left to dry at room temperature.

24. The DNA pellet was dissolved overnight at 4°C in sterilised distilled water (the volume depends on the yield).

25. The quantity and purity of DNA was determined UV spectrophotometrically. The OD at 260nm gives an indication of the quantity. The ratio of the OD at 260nm and 280nm (OD_{260}/OD_{280}) gives

an indication of the purity. A ratio of 1.8 or greater indicates a pure preparation of DNA, a ratio significantly less than 1.8 indicates contamination with protein.

25. The DNA sample was aliquoted into labelled 0.5ml centrifuge tubes and stored at -20°C.

2.3 DNA EXTRACTION USING NUCLEON.

1 Pour blood (about 5-10 ml) into a 50 ml Falcon tube. Add reagent A (10 mM Tris-HCl, 320 mM sucrose, 5 mM $MgCl_2$, 1% Triton X-100; adjust to pH 8.0 with 2M NaOH) mix by vortexing, and allow to stand for 3-4 min.

2. Spin 2300 rpm, 4 min, and discard supernatant.

3. Add 40 ml reagent A, resuspend pellet by vortexing thoroughly, allow to stand for 4 min and repeat step 2.

4. Add 2.5 ml reagent B (400 mM Tris-HCl, adjust to pH to 8.0 with 2M NaOH, 60 mM EDTA, 150 mM NaCl, 1% SDS).

5. Vortex thoroughly to resuspend the pellet and pour into a 5 ml tube. Add

700μl sodium perchlorate (5M) and rotary mix at room temperature for 15 min.

6. Incubate 25 min, 65°C, and invert at least twice during this time.

7. Add 2.5 ml chloroform (kept at -20°C). Rotary mix for 10 min, then centrifuge at 2,500 rpm for 1 min.

8. Add 400μl nucleon silica suspension drop by drop, do not mix, then centrifuge at 4,000 rpm for 3 min.

9. Decant the upper layer into a new 5 ml tube, and centrifuge at 4,000 rpm, 2 min.

10. Remove the solution into a new 50 ml Falcon tube and add two volumes of cold absolute ethanol. Incubate at -20°C, 15 min, then pick out DNA using a hooked Pasteur pipette, place in 200μl sterile distilled water, and allow to resuspend overnight.

11. Read absorbance at 260 and 280 nm. Use 10μl DNA solution in 290μl water. Multiply value at 260 nm by 1.5 to get DNA concentration in μg/μl.

2.4 THE POLYMERASE CHAIN REACTION (PCR).

2.4.1 Introduction.

PCR is an in vitro method of nucleic acid synthesis by which a particular segment of DNA can be specifically replicated (Saiki et al, 1985; Mullis and Faloona, 1987). The reaction requires double-stranded template DNA, synthetic oligonucleotide primers, deoxynucleotide triphosphates to provide both energy and nucleotides for DNA synthesis, DNA polymerase, and reaction buffer containing magnesium ions. It involves repeated cycles of denaturation of the double-stranded DNA, annealing of the primers to their complimentary sequences, and extension of the annealed primers with DNA polymerase. The primers are designed to anneal to opposite strands of the DNA so that they flank the target segment. They dictate the direction of synthesis, and guide the polymerisation to produce two copies of the target segment from one double-stranded DNA template. The resultant copies are complimentary and are capable of binding primers, they will themselves act as template for further amplification. Thus successive cycles will theoretically double the amount of target DNA synthesised in the previous cycle. This results in the exponential amplification of the specific target segment, approximately 2^n, where n is the number of cycles of amplification completed.

2.4.2 Preventing Contamination.

PCR is efficient, and has the potential to produce 1 x 10^6 copies from a single copy of target DNA. There is therefore the possibility of contamination producing a false-positive amplification. Airborne contamination such as sloughed off skin cells or hair roots are ordinarily not a problem. A minute contribution of foreign DNA to the reaction is unlikely to have much effect, unless a single cell is being used as target DNA. Under normal conditions the greatest risk comes from the introduction of previously amplified DNA into the new reaction.

The following steps are taken to avoid contamination:

1. Sterilisation of pipette tips, tubes and reagents.

2. Separate pipettes were designated for preparing PCR reagents and reactions, and were kept away from areas where products were handled.

3. An area was designated for preparing PCR reactions, which was away from areas for DNA extractions, and the handling of PCR products.

4. Reagents and primers were aliquoted.

5. To minimise the handling of the solutions and reduce the number of pipetting steps, a "master mix" was prepared when many identical amplifications were to be performed.

6. For each 'master mix', a water blank containing no DNA was run with every PCR amplification to test for the absence of contamination.

2.4.3 Design of oligonucleotide primers.

The design of primers is empirical. There are no "hard and fast" rules that will produce an efficient primer pair, although there are some general guidelines:

1. If possible, the average G and C content of the primers should be around 50% with a random base distribution.

2. The primers are checked for complimentarity. Particular attention is paid to avoid primers with 3' overlaps, thus avoiding "primer-dimer" artefacts.

3. Primers were designed 20 - 30 bases in length.

2.4.4 Visualisation of PCR product.

After completion of PCR, the product was run out on a 1.5% agarose gel to

ensure that amplification of the target DNA had occurred.

1. A 100ml gel was prepared with Seakem agarose (Flowgen) in 1 x TBE buffer in a conical flask. Ethidium bromide (0.4μg/ml) was added to the agarose solution and mixed by gentle swirling of the flask. The gel was poured and allowed to set with the combs inserted.

2. The gel was submerged in an electrophoresis tank (Flowgen) filled with 1 x TBE buffer.

3. 2μl of Bromophenol blue loading dye was pipetted into 1.5ml tubes.

4. 6μl of PCR product from each sample was then pipetted into a separate tube with the loading dye.

5. The 8μl of sample was then loaded into the wells of the gel.

6. The gel was run at 150V (10V per cm) for 30 minutes.

7. The DNA fragments were then visualised on a UV transilluminator (Vilber Lourmat Ltd) and photographed with a Polaroid CU5 camera. The photograph was kept for documentation.

2.5 MICROSATELLITE MARKERS.

2.5.1 Introduction.

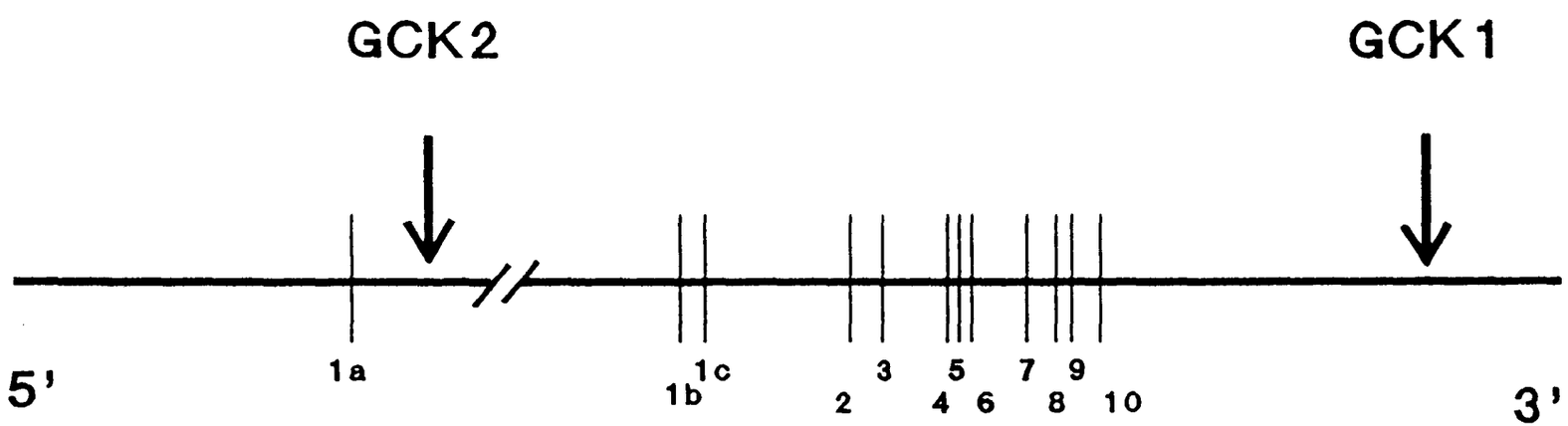
Within the genome are interspersed repetitive DNA sequences which have the form of a dinucleotide repeat such as $(CA)_n$ and $(GA)_n$, trinucleotide repeats and tetranucleotide repeats. These DNA sequences have been shown to exhibit length polymorphisms which are termed microsatellite polymorphisms (Weber and May 1989; Litt and Luty 1989; Economou 1990).

Microsatellite polymorphisms can provide important information as they may be used as genetic markers if they are found to be linked to a particular gene of interest. These microsatellite polymorphisms can be analysed once the sequence surrounding them has been determined. PCR primers may then be designed which are unique to the region flanking the repeats. The region of DNA can then be amplified and the sizes of the amplified fragments can be determined by running them on non-denaturing polyacrylamide gels.

2.5.2 Microsatellite polymorphisms of the glucokinase gene.

To study the glucokinase gene we used two microsatellite polymorphisms, GCK1 and GCK2, which flank the gene (Figure 2.5.2.1). GCK1 is a repeat sequence of the form $(CT)_n$ $(CA)_n$ approximately 10kb 3' to the glucokinase gene. GCK2 is a

Figure 2.5.2.1 Schematic diagram showing the organisation of the glucokinase gene and the position of the microsatellite polymorphisms GCK1 and GCK2.



Exons of the glucokinase gene are indicated by vertical lines and numbers.

repeat sequence of the form $(CT)_n$ $(CA)_n$ approximately 6kb 5' to the glucokinase gene.

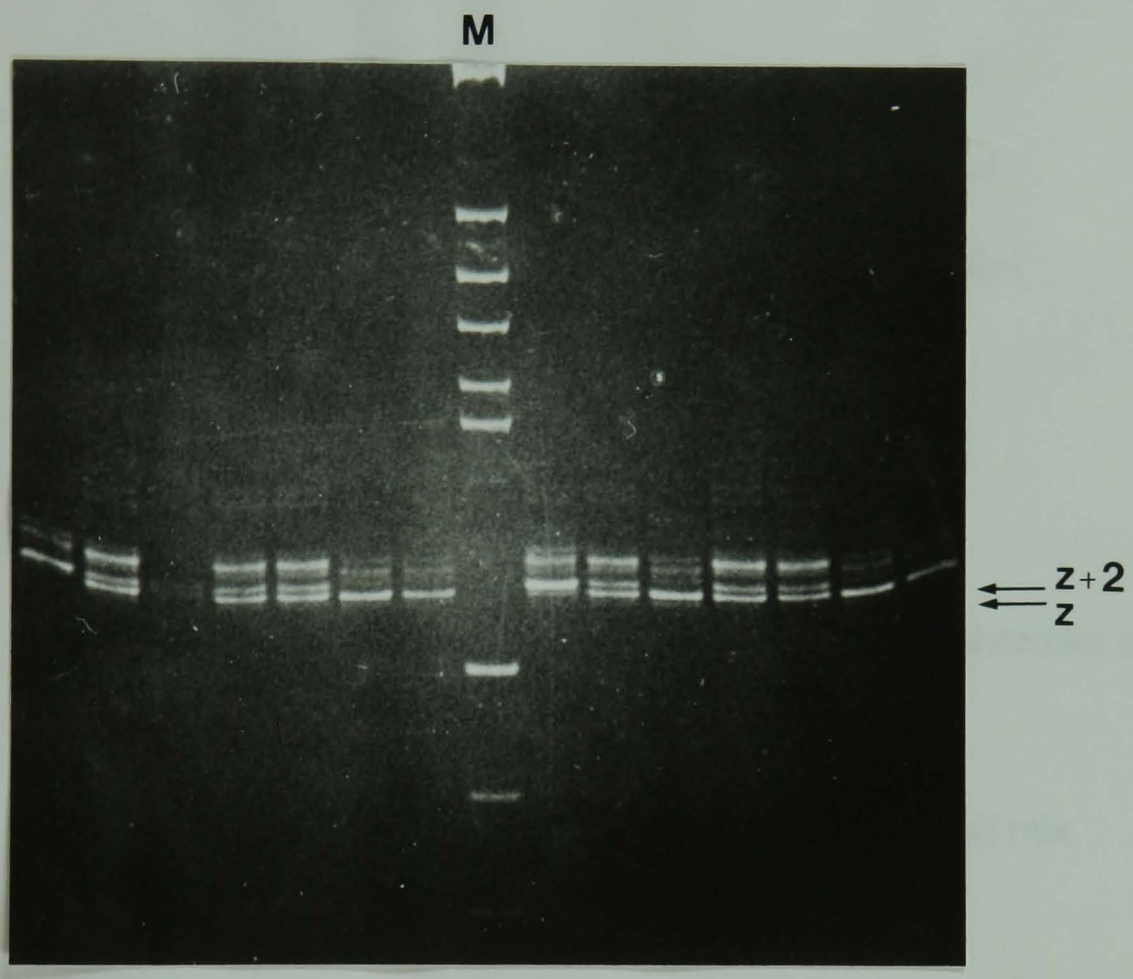
PCR amplification was performed using the following primers:

GCK1	9509	5' TTG GTC AGT GTA GGC TGA ACT CAT G 3'
	9510	5' CCC ACA CCA AAA CTG CCT GTA TTA G 3'
GCK2	hGK-CA1	5' AAC AGA TAC GCT TCA TCC TG 3'
	hGK-CA2	5' TGT CTG CAA CTT ACT CTT AC 3'

After PCR amplification, and conformation of product, sample was loaded onto an 8% polyacrylamide gel. Generally 6μl of sample was added to 3μl of bromophenol blue dye. If the PCR product is weak or the bands visualised on the polyacrylamide are too feint to score and have to be repeated, more product can be loaded.

To aid scoring of alleles, a molecular weight marker is run, or a sample of a known genotype. Alternatively sample from pedigree members may be run alongside each other and the relationship of the alleles assessed when scoring. Figure 2.5.2.2 shows alleles of GCK1.

Figure 2.5.2.2 Agarose gel stained with ethidium bromide and showing alleles of GCK1.



M - molecular weight marker.

2.6 Polyacrylamide gel preparation.

10 X TBE	3ml
distilled water	21.ml
19:1 acrylamide/bis	6ml
TEMED	24μl
25% ammonium persulphate	190μl

1. Clean glass plates with ethanol and distilled water.

2. Arrange glass plates in BioRad Protean II electrophoresis apparatus.

3. Add gel components together in a glass beaker and mix.

4. Pour gels between glass plates of Protean II system using a 50ml plastic syringe.

5. Gels are left for 2 hours to polymerise at RT

6. Pre-run gels at 100V for 30 minutes.

7. Load samples and run-in at 200v for 10 minutes.

8. Adjust current to 100V and run for 18 hours.

9. Alleles were detected by ethidium bromide staining (150μl of 1.1M ethidium bromide diluted in 500ml of distilled water) for approximately 15 minutes.

10. Alleles were visualised with UV light (Vilber Lourmat, Ltd.), and photographically recorded using a CU5 Polaroid camera and film.

11. Samples were then assigned with alleles of the microsatellite polymorphisms.

2.7 RESTRICTION ENDONUCLEASES.

Restriction endonucleases are enzymes which cut DNA at a specific recognition sequence. They are used in restriction fragment length polymorphism (RFLP) analysis.

The following restriction endonucleases were used in my work:

Restriction endonuclease	Recognition site	Application
Hha 1 (or Cfo 1)	GCG/C	To identify the missense mutation $Gly^{299} \rightarrow Arg$ in exon 8 of the glucokinase gene.
Pst 1	CTGCA/G	To divide exons 5 and 6 of the glucokinase gene after PCR. These exons are amplified together as they are too close to design primers to amplify them individually.
Sph 1	GCATG/C	To divide exon 9 of the glucokinase gene into two fragments (205bp and 162bp) after PCR, as the product of 367bp is too large to satisfactorily run on an SSCP gel.
Apa 1	GGGCC/C	Used to identify the $tRNA^{(LeuUUR)}$ at position 3243bp of mitochondrial DNA.
BstEII	G/GTNACC	Used to identify the point mutation $Gly^{40} \rightarrow Ser$ in the glucagon receptor (GCG-R) gene

2.7.1 Reaction mixture for Hha 1, Pst 1 and Sph 1 (may be made as a master mix).

Per sample:

distilled water	7μl
10 X reaction buffer	2μl
10U/μl restriction enzyme	1μl

Then add 10μl of PCR product, and incubate at 37°C for 2 hours to allow digestion.

For identifying the exon 8 mutation with Hha 1, digestion product was visualised on a 2% agarose gel with ethidium bromide staining. 10μl of digestion product was added to 4μl of bromophenol blue dye. The total volume of 14μl loaded onto the gel, and run at 10V/cm until adequate separation had occurred. PCR product from a member of Pedigree BX possessing the mutation was digested and run as a positive control. The gel was visualised under UV light and photographically documented.

2.7.2 Reaction mixture for Apa 1 (may be made as a master mix).

Per sample:

distilled water	5μl
10 X reaction buffer	2μl
20U/μl of Apa 1	1μl

Then add 10μl of PCR product and incubate at 37°C for 2 hours.

4μl of bromophenol blue was added directly to the digestion reaction and the total volume of 24μl was loaded onto a 3% gel with ethidium bromide staining. Positive control (provided by AE Harding) was run with the samples.

2.7.3 Reaction mixture for BstEII (may be made as a master mix).

Per sample:

distilled water	5μl
10 X reaction buffer	2μl
10U/μl of BstEII	1μl

Then add 7μl of PCR product and incubate at 60°C for 2 hours. The samples were then loaded onto a 3% agarose, 1% NuSieve gel with ethidium bromide staining and electrophoresed. Digestion products were visualised using UV-light.

2.7.4 Restriction analysis to detect the glucokinase missense mutation $Gly^{299} \rightarrow Arg$.

1μg of genomic DNA was amplified by PCR using the primers:

8c 5' TCC CGG CTT CCA CCT GCA TGA 3'

8d 5' GTG GAA GAG CAG GTT TTC GTC 3'

and cycling conditions of:

Initial denaturation	96°C	3mins
35 cycles of	94°C	15secs
	60°C	15secs
	72°C	20secs
Final extension	72°C	10mins

The product was 159 bp . After confirming amplification, 10μl was digested with 10Units of Hha 1 (or Cfo1) for 2 hours at 37°C. Fragments were resolved on a 2% agarose gel with ethidium bromide staining.

2.7.5 Restriction analysis to detect the mitochondrial DNA mutation of $tRNA^{(LeuUUR)}$ at position 3243bp.

1μg of genomic DNA was amplified by PCR using the primers:

MITFS	5' AAG GTT CGT TTG TTC AAC GA 3'
MITRS	5' AGC GAA GGG TTG TAG TAG CC 3'

and the cycling conditions of:

Initial denaturation	96°C	4mins
30 cycles of	93°C	30secs
	52°C	30secs
	72°C	40secs
Final extension	72°C	10mins

The product was 428bp. After confirming amplification, 10μl was digested with 20Units of Apa1 for 2 hours at 37°C. Fragments were resolved on a 3% agarose gel with ethidium bromide staining. Presence of the mutation produces restriction fragments of 315bp and 113bp.

2.7.6 Restriction analysis to detect the point mutation $Gly^{40} \rightarrow Ser$ in the glucagon receptor gene.

1μg of genomic DNA was amplified by PCR using the primers:

FS	5' TGT CTG GTT GCT TGT GCA TG 3'
RS	5' GAA GAG AAC TCA GGA AGT GC 3'

and the following cycling conditions:

Initial denaturation	94°C	5 mins
32 cycles of	94°C	90 secs
	55°C	1 min
	72°C	30 secs
Final extension	72°C	10 mins

The product was 196bp. After confirming amplification, 5μl was digested with 5U of BstEII at 60°C for 2 hours. Fragments were resolved on a 3% agarose, 1% NuSieve gel with ethidium bromide staining. Digestion product was visualised using UV-light. Absence of the mutation results in digested fragments of 108bp and 88bp. Presence of the mutation removes a cutting site. The heterozygous mutation results in fragments of 196bp, 108bp and 88bp being visualised.

2.8 MUTATION SCREENING USING SINGLE-STRANDED CONFORMATIONAL POLYMORPHISMS (SSCP).

2.8.1 Introduction.

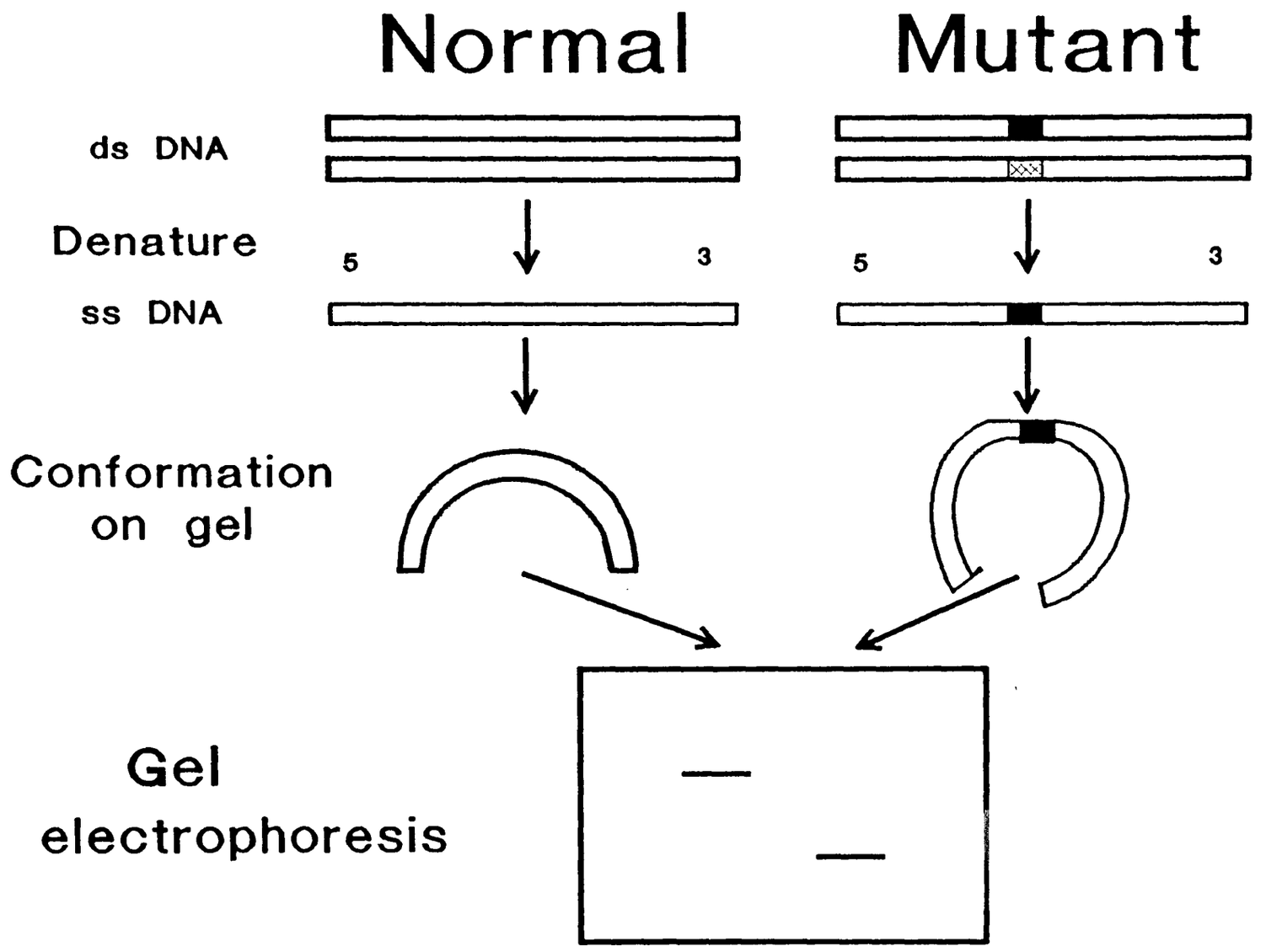
Screening for mutations prior to sequencing can reduce the time and costs of identifying mutations. This technique detects mutations as single-stranded conformational polymorphisms, with the possibility of distinguishing a single base change.

In non-denaturing conditions single-stranded DNA has a folded secondary structure (conformation) which is determined by intramolecular interactions and therefore its amino acid sequence (primary structure). When electrophoresed on a non-denaturing polyacrylamide gel, the single-stranded DNA will have a specific mobility depending on this secondary structure. Any difference in the amino acid sequence of a DNA sample, due to a mutation or polymorphism, will be detected as a mobility shift and will produce a different band pattern when compared to the wild-type (Figure 2.8.1).

The conformation of the single-stranded DNA may be altered by a number of conditions, these have to be optimised to detect the mutations:

1 Properties of gel-pore size determined by percentage of

Figure 2.8.1 Principles of mutation screening using the technique of single-stranded conformational polymorphism (SSCP) analysis.



polyacrylamide, ratio of acrylamide/bis (cross-linker), and temperature at which polymerisation is carried out, including temp. of reagents.

2. Presence and percentage of glycerol; has a weak denaturing action which partially opens the folded structure so that more surface area is available for gel to "sense" conformational changes.

3. Temperature at which electrophoresis is carried out.

4. pH of buffer used to make gel.

5. pH and Ionic strength of buffer system.

Optimal conditions have been developed for each of the glucokinase exons 2-10 (Chapter 5)

2.8.2 Method.

Amplify exon under investigation using relevant primers and PCR.

Run PCR product on a 1.5% agarose gel to check amplification.

2.8.3 Preparation of polyacrylamide gels.

For SSCP denatured samples are run on polyacrylamide gels at two different conditions, generally, 10% polyacrylamide gel with 5% glycerol at 25°C, and 10% polyacrylamide gel without glycerol at 4°C.

Ensure distilled water is used for SSCP as chloride ions interfere with silver staining.

For one gel the following volumes are used:

	4°C	25°C
10X TBE	3ml	3ml
Distilled water	19.5ml	18ml
5% glycerol	------	1.5ml
Acrylamide/Bis	7.5ml	7.5ml
TEMED	24µl	24µl
25% Ammonium Persulphate	190µl	190µl

The gels are made using fresh 10X TBE and 25% ammonium persulphate.

The gels are poured at RT and allowed to polymerise for at least 2 hours prior to use.

Electrophoresis is carried out in 1X TBE buffer. Prior to loading of samples the gel is run at 350V for 30 minutes.

2.8.4 Sample preparation.

SSCP loading dye: Bromophenol blue, xylene cyanol, and formamide. Formamide prevents the renaturation of the single strands of DNA after denaturation.

Double stranded DNA (DS) is run with the samples to act as a marker. A positive control (PC) is also loaded onto each gel if available. The loading position of the DS and PC aids identification and orientation of the gel.

Samples are prepared as follows in labelled PCR reaction tubes.

Sample tubes: 16μl dH_2O + 2μl SSCP dye + 2μl PCR product

PC tubes: 16μl dH_2O + 2μl SSCP dye + 2μl PCR product

DS tubes: 17μl dH_2O + 2μl SSCP dye + 1μl dsDNA

The tubes are capped centrifuged for a few seconds at 4°C and then placed in the PCR machine.

Samples are denatured at 95°C for 6 minutes. To prevent renaturation they are immediately placed on ice for 10 minutes. After this they are centrifuged for a few seconds at 4°C, and returned to ice.

1μl of double-stranded DNA is then added to the DS tubes.

The samples are then loaded onto the polyacrylamide gel using a duck-billed pipette tip.

The gel is typically run at 350V for approximately 18 hours, but the voltage may be altered to vary the run-time and separation.

2.8.5 Silver staining of gels.

Bands are visualised by silver staining (BioRad). Clarity depends on the amount of sample loaded. Gel may have a dark background or be stained totally black due to chloride ions in the water used to make the solutions etc. The supplier's protocol is followed, except that excess oxidiser is removed with 4 x 5 minute washes with water

SSCP is not infallible. May gain false positives or negatives, and have problems with reproducibility.

2.8.7 Mutation screening of the glucokinase gene using SSCP.

PCR primers:

Exon 2		5' CCA GCC CGA CTG CTC CCA TCC 3'
		5' CTT CTG GAT GAG GAG CCG GTT 3'
Exon 3		5' TAA TAT CCG GCT CAG TCA CC 3'
		5' CTG AGA TCC TGC ATG CCT TG 3'
Exon 4		5' TAG CTT GGC TTG AGG CCG GTG 3'
		5' TGA AGG CAG AGT TCC TCT GG 3'
Exons 5&6[a]		5' GCA GCC ACG AGG CCT ATC TC 3'
		5' GAG CCT CGG CAG TCT GGA AG 3'
Exon 7		5' AGT GCA GCT CTC GCT GAC AG 3'
		5' CAT CTG CCG CTG CAC CAG AG 3'
Exon 8	8 2a	5' TGC CTG CTG ATG TAA TGG TCC 3'
	8 b	5' TGA GAC CAA GTC TGC AGT GC 3'
Exon 9[b]		5' ACT GTC GGA GCG ACA CTC AG 3'
		5' CTT GGA GCT TGG GAA CCG CA 3'
Exon 10		5' GTC GAC TGC GTG CAG GGC GC 3'
		5' TGT GGC ATC CTC CCT GCG CT 3'

[a]Exons 5&6 are amplified together and then digested with Pst1 to obtain the two exons:

[b]Exon 9 is amplified and the 367 bp product is digested with Sph 1 to obtain 205 and 162 bp fragments.

Master mix for digestion with restriction endonuclease:

H_2O	7μl
10X Buffer	2μl
Enzyme	1μl
PCR Prod	10μl

Use sterilised pipette tips, tubes, covers and dH_2O. Ensure that enzyme and buffer are thawed. Thoroughly mix 10X buffer. Gently vortex master mix prior to use.

Incubate at 37°C for 1.5 hours.

When run on SSCP gel load twice as much sample as normal (ie 4μl sample, 2μl dye and 14μl dH_2O)

Amplify exon of interest using the above primers.

Initial denaturation	96°C	4 mins
35 cycles of	94°C	30 secs
	62°C	30 secs
	72 °C	40 ssecs
Final extension	72°C	10 mins

PCR Master Mix for a 50μl reaction vol.

H_2O	33μl
10X buffer	8μl
dNTP mix	8μl
Primer 1 (25pM)	0.25μl
Primer 2 (25pM)	0.25μl
Taq	0.5μl

SSCP- samples are run at two conditions (Chapter 5, Table 5.4).

2.9 SEPARATION OF PCR AMPLIFICATION PRODUCTS USING MAGNETIC DYNAL BEADS IN PREPARATION FOR DIRECT SEQUENCING.

2.9.1 Requirements:

1 Dynal Magnetic Particle Concentrator (MPC-E) from Dynal AS, Ltd., N-0112 Oslo, Norway.

2 Dynalbeads M-280 Streptavidin.

3 TES (autoclaved): 10mM tris-HCl, 1mM EDTA, 100mM NaCl.

4 0.15M NaOH (made fresh daily; prepare 1M stock in sterile water).

5 Sequenase kit Version 2.0 United States Biochemical.

2.9.2 Preparation of single-stranded template.

1. PCR is performed in a 100µl reaction; one of the primers is 5' biotinylated. The PCR conditions have to be such that no non-specific products are produced. After confirmation of amplification, all the remaining volume is used in the subsequent procedures.

2. Resuspend Dynalbeads thoroughly prior to use.

3. Take 50μl of beads per 20pmole of biotinylated primer and place into a 0.5ml eppendorf tube.

4. Resuspend required volume of Dynalbeads with 100μl of TES. Gently mix by pulling suspension up and down yellow tip with the pipette. No vortexing.

5. Place the eppendorf tube into the Dynal MCP-E rack at RT with the hinge of the tube facing the back of the block. Leave for a minimum of 30 seconds to allow the beads to be bound to the bottom of the tube.

6. Carefully remove supernatant from the bottom of the tube and discard. Then remove tubes from MCP-E and place in a rack on the bench

7. Repeat steps 4,5 and 6.

8. Resuspend Dynalbeads by gentle repeated flushing with 100μl TES, using the pipette.

9. Mix prewashed beads to all of the remaining PCR product. Mix gently using the pipette and leave in a rack on the bench for 5 minutes; give the

tubes an occasional gentle tap. This allows the double-stranded DNA to absorb to the beads.

10. Place tube into MCP-E for 30 seconds. Remove supernatant and discard it.

11. Add 100µl of 0.15M NaOH to the tube, mix gently with the dsDNA and beads and leave on the bench for 5 minutes to allow denaturation of the DNA. The biotinylated strand will be retained on the beads and the non-biotinylated strand will be in the solution.

12. Place tube in MCP-E. Remove the supernatant containing the non-biotinylated ssDNA and place in a new eppendorf tube. Take tube from MCP-E and place in rack on the bench.

13. Add 1/10th volume of 3M Sodium Acetate pH5.6 and 2.5 volumes of ice-cold ethanol. Place in freezer for 5 minutes.

14. Remove tube from freezer and centrifuge. Wash with 80% ethanol, allow to dry. Resuspend pellet in 10-20µl of distilled water. This non-biotinylated strand is now ready for direct sequencing. Store at -20°C until required.

15. Resuspend the Dynalbeads (with the biotinylated ssDNA) in 100μl TES; wash by gentle mixing using the tip and pipette.

16. Place tube in the MCP-E, remove supernatant and discard. Place tube in rack on bench.

17. Repeat steps 15 and 16 but use 100μl of distilled water rather than TES.

18. Resuspend Dynalbeads and biotinylated DNA in 20μl of distilled water and leave at -20°C until required for sequencing.

2.10 SEQUENCING SINGLE-STRANDED DNA.

2.10.1 General.

Perform standard dideoxy sequencing protocol with USB Sequenase kit version 2.0. Sequencing primers are preferably internal to the ones used for PCR amplification, otherwise the same primers as those used for PCR can be used.

For ssDNA template generated with forward PCR primer, use reverse internal sequencing primer. For ssDNA template generated with reverse PCR primer, use forward internal primer.

2.10.2 Annealing.

Sequencing primer anneals to ssDNA template.

Annealing reaction:

template ssDNA	5μl (max 7μl, adjust volume of distilled water added).
5x reaction buffer	2μl
sequencing primer (2pmol)	1μl
distilled water	2μl (adjust in relation to template ssDNA)
TOTAL	10μl

1. Place components of annealing mixture into a sterile 0.5ml eppendorf tube.

   Heat at 68°C in hot block and incubate for 2 minutes.

2. Remove block from heater, and allow to cool on the bench to RT.

3. Place tubes on ice until labelling is carried out.

2.10.3 Preparation for the next steps.

1. Thaw the following on ice:

   GTP labelling mix

   DTT

   α^{35}S dATP

   enzyme dilution buffer

   STOP solution

   ddNTP termination solutions

2. Ensure the solutions are completely thawed before proceeding with the next steps.

3. Prepare 4 tubes labelled with sample ID and G, A, T and C

   Add 2.5µl of ddNTP termination solution to the respective tube. Prewarm at 37°C in hot block.

4. Dilute dGTP labelling mix to 1:10

5. Dilute Sequenase 1:8 with enzyme dilution buffer (use within 60 minutes of preparation).

2.10.4 Labelling.

1. Prepare a master mix (containing DTT, dilute dGTP, α^{35}S dATP and dilute Sequenase) according to the number of samples.

Reaction mix per sample:

DTT (0.1M)	1μl
dGTP (diluted 1:10)	2μl
α35S dATP	0.5μl
Sequenase enzyme (diluted 1:8)	2μl
TOTAL VOLUME	5.5μl

2. Mix by gentle pipetting back and forth. Add reaction mixture to the tube containing the 10μl of template/primer complex. Incubate on bench at RT for exactly 3 minutes.

2.10.5 Chain termination reaction.

1. When the labelling is complete, quickly and gently mix the solution.

2. Aliquot into four 3.5ml volumes and add one into each of the four tubes containing the termination mix kept at 37°C, by carefully placing the 3.5ml volume as a drop onto the side of the eppendorf tube.

3. When all four tubes have received an aliquot, remove them from the hot block, place in a mini-centrifuge and spin them for a couple of seconds.

4. Remove the tubes from the centrifuge and replace them in the 37°C hot block. Incubate them for <u>exactly 3 minutes.</u>

5. Remove the tubes from the hot block and place them in a mini-centrifuge. Add 4μl of STOP solution to the side of each tube. Spin the tubes for a couple of seconds.

6. The samples can now be stored at -20°C until required to run on the sequencing gel.

2.10.6 Sequencing gel.

Use a 6% 7M urea denaturing gel.

Require a pair of siliconised glass gel plates; one of the pair has a set of lugs.

1. Mark side to be siliconised with a diamond scratcher.

2. Wash plates with ethanol, then hot tap water, then distilled water.

3. Place glass plates in flow hood with marked side facing upwards. Pour silicon (BDH UN No. 2810 - Merck Ltd., Prod. 33164: dimethyldichlorosilane solution. 2% in 1,1,1-) and wipe with a towel to evenly distribute over entire surface.

4. Repeat once, then allow to dry, preferably in an oven at 110°C.

5. Repeat step 3 when necessary, generally every 5-10 gels.

6. Before every use, repeat step 2.

7. With the siliconised surface facing upwards, place plastic spacers along both long sides of the glass plate which does not have the lugs.

8. Place the other glass plate on top with the siliconised side facing downwards.

9. Seal sides and bottom of glass plates well with gel sealing tape (Sigma).

10. Hold the sides of the glass plates with clamps.

11. With lugged plate uppermost, incline the plates so that the open end with the lugs is higher than the bottom.

2.10.7 Gel solution.

Prepare the following as a stock solution:

19:1 acrylamide:bis 40%	75ml
10 x TBE	50ml
Urea 7M	210g (salt)
distilled water	up to 500ml

Stir until dissolved; add a small amount of water at a time, ensuring the total volume of the solution does not exceed 500ml. Store at 4°C until required.

To prepare gel:

1. Take 40ml of stock solution and add the following:

10% ammonium persulphate	400µl
TEMED	30µl

Stir well. Solution will begin to set rather quickly.

2. Take up 30ml in a syringe and pour into one corner between the gel plates. Lift plates to allow gel solution to flow between the plates, down one side and across the bottom. Continue to pour gently until all the volume between the glass plates is full, ensuring that no air bubbles are trapped.

3. Place toothed comb into the gel at the top of the opening between the glass plates with the teeth facing upwards, push in to approximately 3-5mm. Cover top of gel with a paper towel soaked with 1 X TBE, then clamp and allow gel to set. After complete polymerisation gel wrap in cling film and store at 4°C until required.

2.10.8 Running of sequencing gel.

1. Remove cling film and clamps from glass plates, and the adhesive tape from the bottom.

2. Gently remove comb and rinse comb and wells with 1 x TBE. Replace comb with the teeth facing inwards and just touching the gel surface.

3. Place glass plates vertically in gel apparatus; the plate with the lugs facing the upper buffer tank. Put metal against the other glass plate and clamp in position.

4. Fill buffer tanks with 1 X TBE.

5. Pre-run gel for 30 minutes at 25W.

6. Denature prepared samples in hot block at 85°C for 2 minutes.

7. Turn off current to gel apparatus and wash wells with 1 X TBE to flush out urea which continuously floats from gel.

8. Load 2.5 - 3 μl of denatured sample using a pipette and a duck-bill tip. Rinse tip with distilled water after loading each sample.

9. Run gel at 25W for various periods of time to obtain adequate separation.

2.10.9 Preparing gel for exposure.

1. Turn off current to gel.

2. Drain buffer.

3. Remove comb, clamps and metal plate.

4. Place glass plates horizontally on towel-covered bench with lugged plate facing upwards. Remove adhesive tape. Slide spacers out.

5. Slowly lift top glass plate off of gel. Gel should adhere to bottom plate.

6. Place glass plate with the gel into a tank. Gently pour a solution of 10% methanol/ 10% acetic acid so that the gel is completely immersed. Gently agitate tank to loosen gel until it floats freely. Allow to fix for 20 minutes.

7. Remove methanol/acid solution and carefully place a sheet of Whatman filter paper on top of gel. Squeeze out air bubbles. Cover loosely with paper towels to absorb excess fixative solution.

8. With the glass plate facing upwards and the gel sandwiched in-between, place filter paper onto surface of gel dryer. Gently lift glass plate leaving

the gel adhered to the filter paper.

9. Apply vacuum and dry at 60 - 80°C for 45 minutes. Dried gel will securely adhere to filter paper.

10. Expose directly to Kodak film in cassette at RT for 4 days.

11. Develop exposed film using automated processor.

2.10.10 SEQUENCING OF THE GLUCOKINASE GENE.

Amplify exon of interest using primers external to those used for SSCP. One of the pair of external primers has 5' biotinylation to enable separation of the dsDNA into ssDNA using Dynalbeads. The original internal primers are then used as sequencing primers.

PCR amplification:

Initial denaturation	94°C	4mins
35 cycles of	94°C	30secs
	62°C	30secs
	72°C	40secs
Final extension	72°C	6mins

PCR Master Mix for a 100µl reaction volume:

H_2O	66µl
10X buffer	16µl
dNTP mix	16µl
Primer 1 (25pM)	0.5µl
Primer 2 (25pM)	0.5µl
Taq Polymerase	1µl

PCR primers:

Exon 3 external		5' CTT GTG CCT TCC CTC CTC CT 3'
	*B	5' TAG ACA GGT GGC ACC TCC CGT 3'
Exon 3 internal	b	5' TCC TCC TCT TTG TAA TAT CC 3'
	a	5' TAA TAT CCG GCT CAG TCA CC 3'
		5' CTG AGA TCC TGC ATG CCT TG 3'
Exon 8 external	*B	5' GGC TTC CAC CTG CAT GAG GG 3'
		5' GCCCTA GTT TCC CGT CCC TG 3'
Exon 8 internal		5' TGC CTG CTG ATG TAA TGG TCC 3'
		5' TGA GAC CAA GTC TGC AGT GC 3'
Exon 9 external		5' TCC CTG GAG AAC GAG AGG CC 3'
	*B	5' ACG AGA AGA GGA CTA CGA AAT 3'
Exon 9 internal		5' ACT GTC GGA GCG ACA CTC AG 3'
		5' CTT GGA GCT TGG GAA CCG CA 3'

*B indicates 5' biotinylation of primer.

A second internal forward primer (b) was designed for exon 3 to overcome problems with the sequencing reaction found when using the original internal primer (a).

CHAPTER 3

LINKAGE ANALYSIS OF THE GLUCOKINASE GENE IN FIVE MODY PEDIGREES USING THE TWO POLYMORPHIC MICROSATELLITE MARKERS GCK1 AND GCK2

3.1 INTRODUCTION.

Maturity onset diabetes of the young (MODY) is a subgroup of Type 2 diabetes that presents from the second decade and has an autosomal mode of inheritance (Tattersall 1974; Fajans 1989) which makes it ideal for linkage studies. Tight linkage of the glucokinase gene on chromosome 7p to Type 2 diabetes has been found in French and British pedigrees with MODY (Froguel et al 1992; Hattersley et al 1992b). These studies utilised the polymorphic microsatellite marker GCK1 (Matsutani et al 1992).

In a large multi-generation Oxford pedigree (BX) with 15 diabetic members, a peak LOD score of +4.60 was obtained at a recombination fraction (θ) of zero. This suggested that a mutation in the glucokinase gene contributed to the diabetes phenotype in this pedigree. Subsequently a missense mutation in exon 8 of the glucokinase gene ($Gly^{299} \rightarrow$ Arg) was identified and shown to segregate with diabetes in this pedigree (Stoffel et al 1992b). The effected members in Pedigree BX were diagnosed either when they were young (in pregnancy or on screening) or when they presented symptomatically in middle or old age; most of them were treated by diet alone.

We investigated five well-characterised UK MODY pedigrees for linkage to the glucokinase gene, including a new multi-generation pedigree (EA) with members from Edinburgh and Australia, using the polymorphic microsatellite markers GCK1

and GCK2 (Matsutani et al 1992; Tanizawa et al ; Nishi 1992). Studies by the French suggested that 56% of MODY pedigrees had diabetes which was caused by defects in the glucokinase gene (Froguel et al 1993).

3.2 AIMS.

To establish whether in these five MODY pedigrees diabetes was linked to the glucokinase gene. Those in whom linkage was excluded could then be used in studies of other candidate genes.

3.3 METHODS.

3.3.1 Subjects.

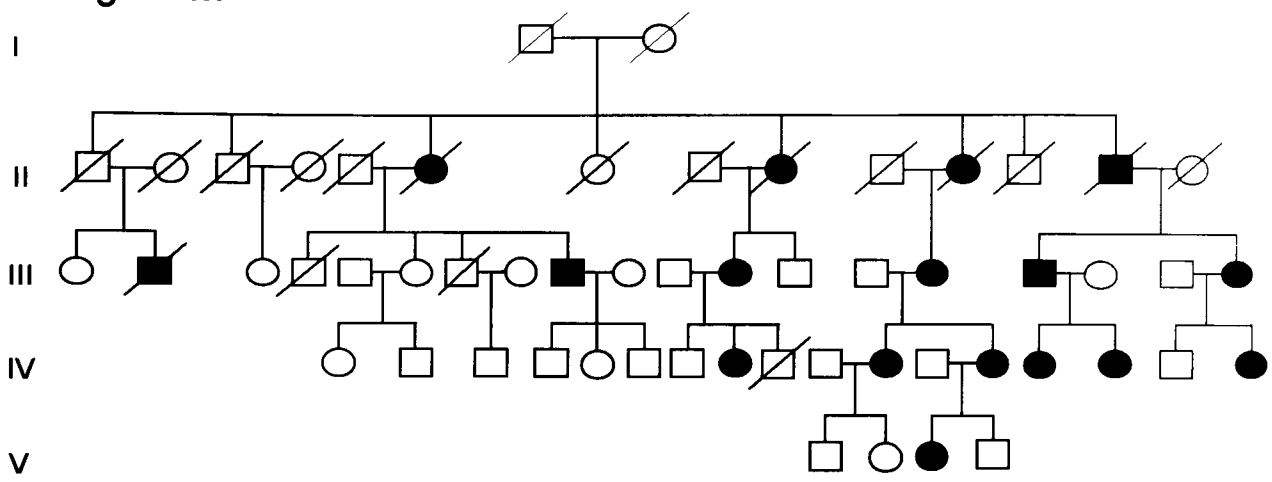
Five white Caucasian MODY pedigrees were studied (Figure 3.3.1), the probands reside in London (M), Oxford (A), Leicester (H), Norwich (N) and Edinburgh (EA). The clinical details are shown in Table 3.3.1. Pedigree M is one of the 3 families first described by Tattersall (Tattersall 1974).

3.3.2 PCR analysis of GCK1 and GCK2.

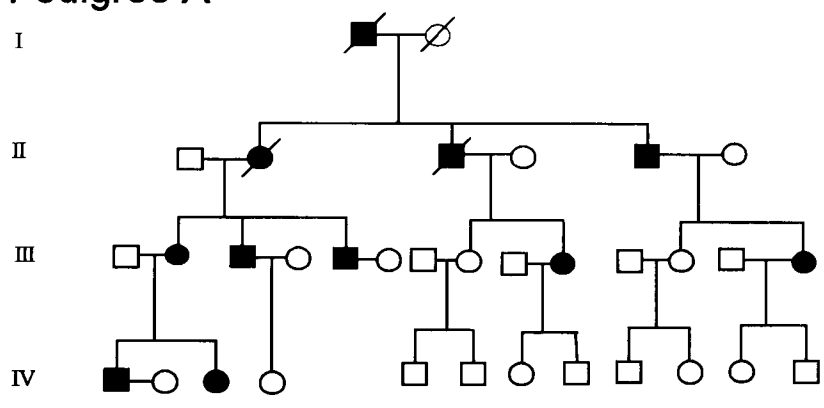
DNA was extracted from peripheral whole blood by standard phenol/chloroform methods (Maniatis et al 1982). Amplification of the two microsatellite

Figure 3.3.1 Maturity Onset Diabetes of the Young (MODY) Pedigrees.

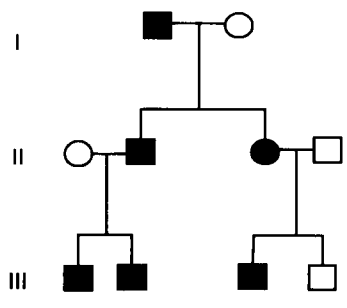
Pedigree M



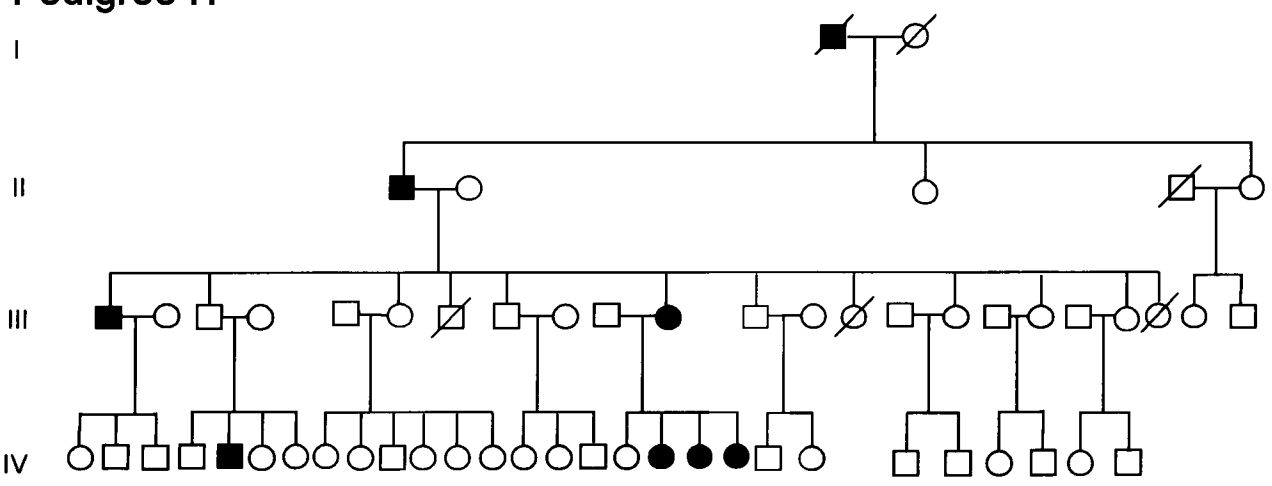
Pedigree A



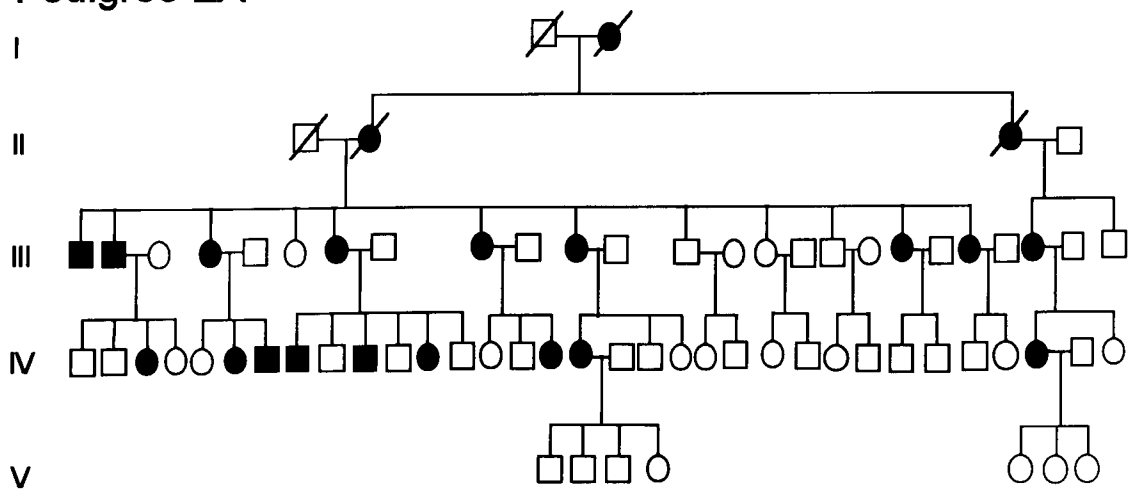
Pedigree N



Pedigree H



Pedigree EA



Key to pedigree trees.

Roman numerals on the left indicate generation number and numbers above the symbol indicate the patient number with that generation.

Symbols are:

squares=males

circles=females

closed symbol=diabetes

diagonal shading (or diagonal cross)=glucose intolerance

open symbol=normoglycaemia

central dot=not tested

slash=dead.

This convention will be used on subsequent pedigrees.

Table 3.3.1 Clinical characteristics of MODY patients in the 5 families and in subjects in pedigrees BX with a glucokinase missense mutation.

		At diagnosis		Current status		
Pedigrees	**Gender M/F**	**Age (years) Median (range)**	**Diet/OHA/ins**	**Age (years) median (range)**	**BMI (kg/m^2) median (range)**	**Therapy diet/OHA/ins**
M	1/8	15(8-28)	6/-/3	29(8-60)	23(21-26)	4/5/-
A	4/3	17(6-37)	1/3/3	38(19-56)	23(22-29)	1/3/3
N	2/2	19(15-20)	2/2/0	35(25-46)	21(21-22)	2/2/0
H	5/4	21(11-50)	5/0/3	26(21-76)	24(23-27)	3/2/3
EA	4/11	19(14-48)	6/4/4	47(29-60)	25(22-33)	0/9/5
Total or Medians	10/28	19(6-50)	20/9/13	35(8-76)	23(21-33)	10/21/11
BX subjects with a GCK mutation	7/11	21(14-63)	16/1/1	41(18-81)	24(18-39)	15/2/1

OHA = Oral hypoglycaemic agents; ins = insulin; HbA1c = Haemoglobin A1c; GCK = glucokinase.

polymorphisms GCK1 and GCK2 was performed using the polymerase chain reaction (PCR). 0.05μmoles of primers, in 50μl reaction volumes containing 0.25μg of DNA, 100μg of each dNTP, 10 mmol/l Tris-HCl (pH3.8), 50mmol/l KCl, 1.5mmol/l and 1.5 Units of BioTaq DNA polymerase. The PCR involved initial denaturing at 95°C for 3 minutes, then 40 cycles of denaturing at 94 °C for 30 seconds, annealing at 55°C for 20 seconds, and extension at 72°C for 20 seconds and a final extension of 72°C for 10 minutes. 6ul of PCR product was electrophoresed on an 8% polyacrylamide gel at 100V for 18 hours to resolve the alleles which were detected by ethidium bromide staining.

3.3.3 Linkage Analysis.

Each family was considered separately and analysed using MLINK from the LINKAGE group of programs (version 5.1) assuming dominant inheritance (Lathrop et al 1984). LOD scores were calculated with a recombination fraction of $\theta = 0$ appropriate for a candidate gene. Liability classes were based on prevalence studies of diabetes in the population and in MODY subjects.

Age related penetrance was modelled on the assumption of fasting plasma glucose screening to detect subclinical disease as 0.60 for <20 years, 0.80 for 21-30 years, 0.90 for 31-40 years, 0.95 for 41-50 years, and 0.99 for >50 years for heterozygosity and 1 for homozygosity at all ages (O'Rahilly et al 1988b).

As the clinical presentation in these families was similar to that for classical Type 2 diabetes, phenocopy rates for each liability class were those of the prevalence of Type 2 diabetes in the UK; 0.0001, 0.001, 0.001, 0.005, and 0.03 respectively.

Allele frequencies for GCK1 are taken as being 0.632, 0.092, 0.270 and 0.007 for z, z+2, z+4 and z+6 respectively; for GCK 2 they are taken as 0.336, 0.592, 0.046, 0.007 and 0.019 for 1, 2, 3, 4 and 5 respectively. These allele frequencies were determined in a British Caucasian normoglycaemic control population (See Chapter 4).

3.4 RESULTS.

In the five pedigrees studied diabetes did not segregate with any particular allele of either genetic marker. The calculated LOD scores are shown in Tables 3.4.1 and 3.4.2. Using GCK1 (Table 3.4.1), Pedigrees H and EA were non-informative for this marker The other 3 pedigrees all had a LOD score less than -2.00 (θ=0) which excludes linkage. The cumulative LOD score for all five pedigrees is -12.38 (θ=0). In two pedigrees it was possible to exclude linkage 1 centimorgan from the marker.

Using GCK2 (Table 3.4.2), Pedigree A was non-informative for this marker. 3 of the other 4 pedigrees had a LOD score less than -2.00 (θ=0) which excludes linkage. In these three pedigrees it was possible to exclude linkage 1centimorgan

Table 3.4.1 LOD scores between diabetes and the glucokinase locus GCK1.

	Recombination fraction (θ)						
	0.0	0.01	0.05	0.1	0.2	0.3	0.4
TOTAL	-12.38	-7.55	-3.95	-2.25	-0.73	-0.19	-0.05
Pedigree M	-5.10	-2.97	-1.53	-0.89	-0.36	-0.15	-0.06
Pedigree A	-3.66	-2.32	-1.09	-0.58	-0.17	-0.01	0.03
Pedigree N	-2.37	-1.09	-0.45	-0.21	-0.04	0.01	0.01
Pedigree H	-0.64	-0.58	-0.41	-0.26	-0.10	-0.03	-0.01
Pedigree EA	-0.62	-0.60	-0.47	-0.30	-0.06	0.00	-0.02

Table 3.4.2 LOD scores between diabetes and the glucokinase locus GCK2.

	Recombination fraction (θ)						
	0.0	0.01	0.05	0.1	0.2	0.3	0.4
TOTAL	-17.51	-12.40	-7.21	-4.86	-2.45	-1.03	-0.25
Pedigree M	-4.23	-3.25	-1.92	-1.29	-0.66	-0.32	-0.11
Pedigree A	0.05	0.05	0.03	0.00	-0.04	-0.04	-0.02
Pedigree N	-2.56	-1.66	-0.92	-0.57	-0.23	-0.09	-0.02
Pedigree H	-1.76	-0.83	-0.23	0.00	0.16	0.17	0.12
Pedigree EA	-9.00	-6.70	-4.17	-3.01	-1.68	-0.76	-0.22

from the marker. In Pedigree H, although formal exclusion is not possible, the finding of a LOD score of -1.76 makes it very unlikely that glucokinase mutations result in diabetes in this pedigree. The cumulative LOD score for all five pedigrees was -17.51 (θ=0).

3.5 DISCUSSION.

Diabetes was not found to be linked to the glucokinase microsatellite DNA polymorphisms in the five pedigrees we investigated, suggesting that the gene responsible for diabetes in these five pedigrees is not at, or close to, the glucokinase gene locus. We have only found linkage of glucokinase with diabetes in one pedigree (BX) (Hattersley et al 1992b) out of six MODY pedigrees. This finding contrasts with the report that glucokinase mutations can account for diabetes in 56% of French MODY pedigrees (Froguel et al 1993). In the UK only 12.5% (1/8) Caucasian MODY pedigrees have diabetes linked to the glucokinase gene (Hattersley et al 1992b, Dow et al 1994, this Chapter 3). These differences may reflect true differences between the two countries, or be due to ascertainment bias.

The five pedigrees studied here have quite severe diabetes which requires treatment with oral hypoglycaemic agents (OHA) and insulin. This contrasts with the phenotype observed in subjects with glucokinase mutations, many of whom have impaired glucose tolerance rather than overt diabetes which tends to be diet

or tablet controlled, most members remain undiagnosed, except in female members who become pregnant, and the sub-clinical diabetes rarely progresses to associated complications (Hattersley et al 1992b; Page et al 1995).

The results of this study further highlight the heterogeneity of MODY. Diabetes in MODY pedigrees has been found to be linked to the ADA marker on chromosome 20q (Bell et al 1991), and glucokinase (Froguel et al 1992; Hattersley et al 1992b) and there is at least one more gene responsible for this subgroup of Type 2 diabetes. The pedigrees studied here have since been investigated for linkage to ADA and other candidate genes which could regulate insulin secretion (hexokinase II) and β-cell sensitivity to glucose (glucagon-like polypeptide-1 receptor, and pituitary adenylate cyclase-activation polypeptide receptor) and linkage with these has been excluded (Zhang et al 1995).

Linkage analysis in pedigrees is a powerful tool for examining the role of candidate genes in the aetiology of inherited diseases with a defined mode of transmission (Ott J 1985). Pedigrees with MODY are particularly useful as they have a clear autosomal dominant mode of inheritance, and an early age of onset which allows ascertainment of affected members over three generations (Tattersall et al 1975; Kobberling et al 1982; O'Rahilly et al 1988a). When performing linkage analysis, the parameters were chosen to take into account the complicating factors of high prevalence of Type 2 diabetes in the general population and the age-dependent penetrance of the MODY gene.

This linkage study has shown that defects in the glucokinase gene are an unusual cause of diabetes in British MODY pedigrees with symptomatic hyperglycaemia. The results further highlight the heterogeneity of MODY. There are at least three genes involved in MODY, but as yet only glucokinase has been identified.

CHAPTER 4

MICROSATELLITE POLYMORPHISMS AT THE GLUCOKINASE LOCUS: A POPULATION ASSOCIATION STUDY IN CAUCASIAN TYPE 2 DIABETIC SUBJECTS

4.1 INTRODUCTION.

Tight linkage of diabetes with the glucokinase (GCK) locus on chromosome 7p has been described in French and British Caucasian pedigrees with Maturity Onset Diabetes of the Young (MODY), a subtype of Type 2 diabetes (Froguel et al 1992; Hattersley et al 1992b). Following these results a nonsense mutation (Vionnet et al 1992) and missense mutations (Stoffel et al 1992a & b) of the glucokinase gene were identified. The missense mutation identified in the British MODY pedigree (BX) has also been found in a late onset Caucasian Type 2 diabetes pedigree (AX) (Stoffel 1992a). This suggests that mutations in the glucokinase gene could contribute to the development of classical Type 2 diabetes. Positive association in American Blacks and Mauritian Creoles support this possibility (Chiu et al 1992 a & b). These studies showed an association between alleles of GCK1 and classical Type 2 diabetes.

4.2 AIMS.

We investigated well-characterised white UK Caucasian Type 2 diabetic subjects and normoglycaemic control subjects with two microsatellite polymorphisms at the glucokinase loci, GCK1 and GCK2, to assess the contribution of the glucokinase gene to Type 2 diabetes. We examined for linkage disequilibrium between the polymorphisms and for an association with diabetes.

4.3 SUBJECTS.

The initial cohort comprised 95 unrelated UK white Caucasian Type 2 diabetic subjects from the Oxford clinic of the UK Prospective Diabetes Study, and 76 normoglycaemic volunteer controls from the same geographical area and ethnic group as the diabetic subjects. The diabetic subjects were all diagnosed after the age of 40 years. The normoglycaemic control subjects were all older than 50 years and had a fasting plasma glucose <5.5 mmol/l. An additional cohort of 56 identically defined Caucasian Type 2 diabetic subjects was examined at the GCK2 microsatellite polymorphism only.

4.4 METHODS.

4.4.1 PCR Analysis of the Microsatellite Polymorphisms GCK1 and GCK2.

DNA was extracted from peripheral whole blood using standard phenol/chloroform methods (Maniatis et al 1982). The two microsatellite polymorphisms (Matsutani et al 1992; Froguel 1992; Tanizawa et al ; Nishi et al 1992) were amplified using the polymerase chain reaction (PCR). This was performed with 0.05 μmoles of the primers in 50μl reaction volumes containing 0.25μg DNA, 100μg of each dNTP, 10 mmol/l Tris-HCl (pH3.8), 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$ and 1.5 units of AmpliTaq DNA polymerase. The PCR involved initial denaturing at 95°C for 3 minutes, then 40 cycles of denaturing at 94 °C for 3 seconds, annealing at 55°C

for 20 seconds, and extension at 72°C for 20 seconds and a final extension of 72°C for 10 minutes.

Resolution of alleles was carried out by electrophoresing product on 20cm 8% polyacrylamide gels. Bands were detected by ethidium bromide staining, avoiding the need for radioactivity. Four alleles were identified for GCK1; z, z+2, z+4, z+6, where z is the commonest allele 195 bp long. Five alleles were identified for GCK2; 1, 2, 3, 4 and 5, which were 141, 139, 137, 135 and 129 bp long respectively. Haplotypes were constructed using the genotyping at the separate polymorphisms; this was possible when a subject was homozygous at one, or both of the marker loci.

4.4.2 Statistical Analysis.

To assess the relationship between the two polymorphisms, linkage disequilibrium between alleles was sought by comparing the frequency of the haplotypes with that expected from allelic frequencies, assuming no linkage disequilibrium.

D was calculated as a measure of disequilibrium between two alleles at 2 separate loci. When there were more than two alleles at a locus, D was calculated using the formula (Weir 1990):

$$D_{uv}=p_{uv} - p_u p_v$$

where:

D_{uv}= is the disequilibrium constant between allele u at polymorphism 1 and allele v at polymorphism 2.

p_{uv} is the observed haplotype frequency with allele u at polymorphism 1 and allele v at polymorphism 2

p_u and p_v are the frequencies of alleles u and v respectively.

To assess whether the disequilibrium between the two alleles significantly differed from zero the following value was used:

$$\frac{nD_{uv}^2}{p_u(1-p_u)p_v(1-p_v)}$$

which is asymptotically distributed as a χ^2 with one degree of freedom under the null hypothesis of D = 0 (Weir 1990).

The allelic and haplotype frequencies in the Type 2 diabetic and non-diabetic control subjects was compared using the χ^2 test. In the analysis the overall

distribution of allelic frequencies, alleles with an expected frequency of 5 or less were pooled. When using χ^2 test to assess individual allelic frequencies and linkage disequilibrium between alleles a Bonferroni correction was made for multiple analyses.

4.5 RESULTS.

All the subjects were unambiguously assigned with each of the microsatellite polymorphisms investigated. 4 alleles were observed for the GCK1 polymorphism, and 5 at GCK2. The heterozygote frequency in the normal population was 0.50 for GCK1, 0.66 for GCK2, and 0.80 for both polymorphisms combined. It was possible to infer unambiguous haplotypes with both markers in 54 of the 76 normoglycaemic control subjects and 80 of the Type 2 diabetic subjects.

4.5.1 Linkage Disequilibrium Between Polymorphisms in Normoglycaemic Control Subjects.

Table 4.5.1 shows the results of linkage disequilibrium between polymorphisms in normoglycaemic control subjects. Allele z of the GCK1 polymorphism was positively associated with allele 1 of the GCK2 polymorphism (D = 0.050, χ^2 = 5.78, $p<0.02$) and negatively associated with the allele 2 (D = 0.046, χ^2 = 4.917,

Table 4.5.1. Linkage disequilibrium between glucokinase microsatellite polymorphisms GCK1 and GCK2.

Haplotype		Observed frequency in controls	Expected frequency in controls	D	χ^2
GCK1	GCK2	(n=108)			
z	1	0.269 (29)	0.219	0.050	5.786 a
z	2	0.417 (45)	0.463	-0.046	4.917 b
z	5	0.009 (1)	0.013	-0.004	0.383
z+2	1	0.019 (2)	0.029	-0.010	0.655
z+2	2	0.074 (8)	0.062	0.012	0.842
z+4	1	0.028 (3)	0.067	-0.039	4.595 a
z+4	2	0.176 (19)	0.142	0.034	3.337
z+4	5	0.009 (1)	0.004	-0.005	0.981

Linkage disequilibrium between glucokinase polymorphisms GCK1 and GCK2 was assessed by the disequilibrium coefficient (D) in 54 normoglycaemic control subjects (108 chromosomes). The number of chromosomes in which the haplotypes were observed are shown in parentheses. Expected frequencies were calculated from the allele frequencies assuming no linkage disequilibrium Statistical analysis by χ^2 is as described in the text a $p<0.02$, b $p<0.05$. When multiple analyses were corrected for there was no linkage disequilibrium.

p<0.05). There was a negative association between the z+4 and allele 1 (D = -0.039, χ^2 = 4.595, p<0.05). However, with the Bonferroni correction for multiple comparisons (n=8) none of these associations remained statistically significant.

4.5.2 Analysis of Haplotype and Allelic Frequencies in Normoglycaemic Control and Diabetic Subjects.

Tables 4.5.2.1 and 4.5.2.2 show the results of allele and haplotype frequencies in the normoglycaemic control subjects and the initial cohort of diabetic subjects. There was no significant differences in the overall and individual frequencies of the alleles of the GCK polymorphism (z, z+4 and others χ^2 = 1.01, D.F. = 2, p =/>0.05) or the combined haplotypes (χ^2 = 4.65, D.F. = 4, p =/>0.1). At GCK2 there was no significant difference in the overall distribution of allele frequencies (1, 2 and others χ^2 = 5.72, D.F. =2, p = 0.057). Allele 1 was less frequent in the diabetic subjects than the normoglycaemic control subjects (22% vs 34%)(1 vs non 1 χ^2 = 7.934, D.F. = 1, p<0.02) and the 2 allele more frequent (70% vs 59%) (2 vs non 2 χ^2 = 4.785, D.F. = 1, p<0.05), but neither of these remained significant when multiple analyses (n=5) were taken into account.

To assess whether there was a weak association at GCK2 which did not reach

significance due to the number of subjects studied, a second cohort of 56 Type 2 diabetic subjects were typed for GCK2. When all 151 Type 2 diabetic subjects typed for GCK2 were analysed the allelic frequency distribution (Table 4.5.1.1) was similar to that found in the controls (1, 2 and others χ^2 = 3.63, D.F. = 2, p=/>0.1). No significant difference was seen in the individual allele frequency of this expanded cohort even without correction for multiple analyses (1 vs non 1 χ^2 = 3.530, D.F. = 1, p>0.05; 2 vs non 2 χ^2 = 3.081, D.F. =1, p>0.05).

Table 4.5.2.1 Allelic frequencies in diabetic and normoglycaemic control subjects for glucokinase microsatellite polymorphisms GCK1 and GCK2.

Allele		Frequency in normoglycaemic controls (n=152)	Frequency in initial diabetic cohort (n=190)	Frequency in expanded diabetic cohort (n=302)
GCK1				
	z	0.632 (96)	0.653 (124)	
	z+2	0.092 (14)	0.063 (12)	
	z+4	0.270 (41)	0.284 (54)	
	z+6	0.007 (1)	0.000 (0)	
GCK2				
	1	0.336 (51)	0.221 (42)a	0.252 (76)
	2	0.592 (90)	0.705 (134)b	0.675 (204)
	3	0.046 (7)	0.042 (8)	0.046 (14)
	4	0.007 (1)	0.010 (3)	0.010 (3)
	5	0.019 (3)	0.017 (5)	0.017 (5)

The number of each allele observed are shown in parentheses. Statistical analysis by the χ^2 showed no significant difference in the overall distribution of allele frequencies in the diabetic subjects (initial and expanded cohorts) compared with the normoglycaemic control subjects at GCK1 and GCK2. Analysing the individual allele, significant differences were only seen between the initial diabetic cohort and the normoglycaemic controls: a $p<0.02$; b $p<0.05$. These were not significant when they were corrected for multiple analyses.

Table 4.5.2.2 Comparison of glucokinase haplotype frequencies in normoglycaemic control and diabetic subjects.

Haplotype GCK1	GCK2	Observed frequency in normoglycaemic controls (n=108)	Observed frequency in diabetic subjects (n=160)
z	1	0.269 (29)	0.194 (31)
z	2	0.417 (45)	0.469 (75)
z	3	0.000 (0)	0.019 (3)
z	4	0.000 (0)	0.006 (1)
z	5	0.009 (1)	0.006 (1)
z+2	1	0.019 (2)	0.000 (0)
z+2	2	0.074 (8)	0.037 (6)
z+4	1	0.028 (3)	0.019 (3)
z+4	2	0.176 (19)	0.231 (37)
z+4	3	0.000 (0)	0.019 (3)
z+4	5	0.009 (1)	0.000 (0)

The number of chromosomes with the individual haplotypes are shown in parentheses. Statistical analysis by the χ^2 test showed no significant differences in the overall, or individual haplotype frequencies.

4.6 DISCUSSION.

We have examined two microsatellite polymorphisms (GCK1 and GCK2) at the glucokinase locus and have found no association with Type 2 diabetes in white UK Caucasian subjects. Use of more than one marker enabled us to define haplotypes which increases the power of population association studies and allows linkage disequilibrium between the alleles of the two markers to be assessed. Haplotypes increases the likelihood of finding linkage disequilibrium with a common mutation and have been applied to beta-thalassaemia (Orkin et al 1982), cystic fibrosis (Estivill 1987), and phenylketonuria (DiLella 1987).

In our normoglycaemic population, the linkage disequilibrium between the two markers was not significant when multiple comparisons were taken into account. This may reflect the sample size, rather than the absence of disequilibrium between these two polymorphisms which are approximately 50 kb apart. We only analysed haplotypes where they could be unambiguously assigned. Any bias from the exclusion of double heterozygotes is likely to be small as only 21% of subjects could not be assigned a haplotype. Using both polymorphisms combined increases the heterozygosity rate to 80%, rather than 50% and 60% for GCK1 and GCK2 respectively.

In this study no association was found with diabetes at GCK1, GCK2, or their combined haplotypes in the initial analysis of 95 Type 2 diabetic subjects and 76

normoglycaemic controls. The association with allele 2 of GCK2 was not significant when corrected for multiple analyses. To assess whether there was a weak association at GCK2 which had not reached significance due to the moderate number of subjects studied we typed a further 56 diabetic subjects at this polymorphism. In this second cohort and the total group of 151 diabetic subjects no significant association was seen. The analysis of a second independent cohort has been recommended prior to publication of positive association results to reduce the possibility of a positive association occurring by chance alone (Peto 1988).

We have not confirmed the previous population associations seen with GCK1 in American Blacks and Mauritian Creoles in UK Caucasians. Our results indicate that a single mutation in the glucokinase locus is not a common cause of Type 2 diabetes in our Caucasian population.

As with all population association studies the data should be interpreted cautiously. An infrequent mutation that was in linkage disequilibrium with GCK1 and GCK2 might be present but would not be detected due to the statistical power. The sample size we used would detect an association with 80% power if 30% of Type 2 diabetic patients had a highly penetrant dominant mutation in tight linkage disequilibrium with a marker allele with a frequency of 0.1 (Cox et al 1989). The statistical power of our study is sufficient to detect an association with a common mutation such as that found in 70% of cystic fibrosis (Karem 1990).

Our findings do not exclude the possibility that several different mutations of glucokinase might contribute to diabetes in a small proportion of Type 2 subjects. Linkage studies in Caucasian pedigrees with several affected members (Cook et al 1992; Dow et al 1994) support the conclusion that mutations in the glucokinase gene are unlikely to be common; weakly positive LOD scores were found in only 2 of 12 pedigrees (Cook et al 1992), whereas 56% of MODY pedigrees are linked (Froguel et al 1993).

A population association study in a Welsh Caucasian population (Tanizawa et al 1993), linkage analysis of Caucasian Type 2 diabetic pedigrees (Cook et al 1992; Dow et al 1994), and mutation screening of the glucokinase gene (Chapter 6; MG Warren-Perry unpublished 1994) support our findings that one major mutation in or near the glucokinase gene is not a major cause of Type 2 diabetes in the UK Caucasian population. Over 20 different glucokinase mutations have been identified which cause diabetes, the majority of which are found in exons 7 and 8 (Gidh-Jain et al 1992, Froguel et al 1993 & StCharles et al 1994).

Positive population associations of diabetes with the microsatellite polymorphisms have been reported in American Blacks, Mauritian Creoles, Dravidian Indians, Japanese and Finns (Chiu et al 1992a & b; Noda et al 1993; McCarthy et al 1993 & 1994a). It is possible that there is a common mutation within these ethnic groups. McCarthy et al and Noda et al point out the phenotypic similarity between Type 2 diabetes in Dravidian Indians and Japanese and subjects with a

glucokinase mutation. The positive population associations are not supported by SSCP analysis in American Blacks, Dravidian Indians, Japanese or Finns. No pathogenic mutations have been found in exons 2-10 of the glucokinase gene in these populations (Chiu et al 1993; Nishi et al 1994; McCarthy et al 1994b). There is the possibility that there is a pathogenic mutation in the promoter region of the gene, or in an intron at a splice-donor site that results in diabetes in these populations. Some data supports a role for a promoter polymorphism (Stone et al 1994), but this is not clear. A mutation in the promoter region would be likely to affect the expression of the gene. Genetic admixture is the most common reason for false positive associations and might explain the results in these groups. This can occur even when every effort is made to match diabetics and controls. The best example of this is seen in the Nauruan population in which the European HLA component reduces the susceptibility of diabetes (Serjeantson et al 1983).

There appears to be a number of potential problems with genetic heterogeneity in the Mauritian study The Mauritian Indians are form several areas of the Indian sub-continent, they were predominantly Hindu (80%) and a proportion of Muslims (13%); no breakdown was given between the diabetic subjects and controls and whether they were matched for religion. There will be differences in ethnic origin between the subjects, although Mauritius is not quite like India where strict segregation occurs between the religious and class groups, some segregation will have occurred. The Creoles are of African and Malagasy ancestry with various amounts of European and to a lesser extent Indian admixture. The authors did

refer to Serjeantson and his studies of Nauruans. No differences in the HLA alleles were found between the Type 2 diabetic subjects and controls in this study. As there was no association found in the Indians, the authors suggested that the Indians are not contributing genetic factors to the Creoles positive association, and that it was more likely to be due to the Black African and European Caucasian components. The Black African component was predominantly from Madagascar and French African colonies and may not itself be homogeneous. The contribution of European Caucasian components to the Creole positive association was not supported by the negative population association studies in two UK populations, although these two populations were both Northern European (Tanizawa et al 1993; this Chapter, 4)

An investigation in Black Americans (Chiu et al 1992b) found the z+4 allele of GCK1 to be an independent risk factor for diabetes. This result was not supported by SSCP screening of 60 random Black Americans from this study to detect variants in subjects with z+4 and non-Z+4 alleles (Chiu et al 1993). A major investigation into the contribution of glucokinase gene mutations to Type 2 diabetes in Dravidians (South Indian) has been conducted which utilised both population association and linkage studies (McCarthy et al 1993). Significant differences in allele frequencies at GCK1 were found with an increase in the z allele and decrease of the z+2 allele. No linkage of the glucokinase gene to Type 2 diabetes was found; this along with the positive association suggests a minor role for a single mutation in the glucokinase gene as a cause of Type 2 diabetes

in this population. An association has been shown with the z+4 allele of GCK1 in a Japanese population (Noda et al 1993). A missense mutation of GCK has been identified in Japanese late-onset Type 2 diabetic pedigrees which would seem to support this (Katagiri et al 1992; Shimada et al 1993). A larger study of the contribution of glucokinase to Type 2 diabetes in Japanese subjects found no association of GCK alleles and haplotypes with diabetes (Nishi et al 1994). In addition, mutation screening of exons 2-10 failed to identify any mutations, and the polymorphisms detected did not associate with Type 2 diabetes. These results suggest that mutations of the glucokinase gene are not a major cause of late-onset Type 2 diabetes in Japanese subjects.

A strong association has been found between alleles at GCK1 and glucose intolerance and diabetes in elderly Finnish men (McCarthy et al 1994a), but this has not been supported by SSCP screening (McCarthy et al 1994b). A previous study in this cohort found differences in HLA haplotypes between diabetic and control subjects and concluded that HLA is a major genetic determinant of Type 2 diabetes (Tuomilehto-Wolf et al 1993). Alternatively, these results could suggest that genetic admixture was present in this population and could explain the positive population association and negative mutation screening.

We have found no association of the glucokinase locus with Type 2 diabetes in a UK Caucasian population. This suggests that one major mutation in or near the glucokinase gene is not a major cause of Type 2 diabetes in the UK Caucasian

population. These results are supported by linkage analysis of UK Caucasian Type 2 diabetic pedigrees and mutation screening of the glucokinase gene. Reviewing the published population association investigations of the contribution of glucokinase gene mutations to Type 2 diabetes reveal the problems involved in conducting such studies. The results need to be analysed cautiously, and used in conjunction with linkage studies and mutation screening to gain a clearer and more accurate picture.

CHAPTER 5

DEVELOPMENT OF SSCP TO ENABLE MUTATION SCREENING OF THE GLUCOKINASE GENE

5.1 INTRODUCTION.

Mutations in the glucokinase gene are a cause of diabetes in subjects with MODY, a subgroup of Type 2 diabetes. We intended to screen subjects with gestational diabetes, particularly those who went on to develop Type 2 diabetes, for mutations in the glucokinase gene and therefore required a simple, rapid and reproducible method. SSCP analysis detects base changes in the DNA sequence as differences in electrophoretic mobility when compared to "wild-type" (Orita et al 1989). SSCP had been shown to be effective in detecting glucokinase mutations in French, British and Japanese pedigrees (Vionnet et al 1992; Stoffel et al 1992b; Katagiri et al 1992), so we decided to use this method.

5.2 AIMS.

To develop single-stranded conformational polymorphisms analysis to screen for mutations of the glucokinase gene in at-risk populations of subjects such as gestational diabetic subjects. This method needed to be sensitive, detecting as many mutations as possible.

5.3 METHODS.

Individual exons, and at least 30 bases of the intron, were amplified using primers modified from those previously published (Stoffel et al 1992a; Chiu et al 1993)

and conditions described in Chapter 2; 2.8.1-2.8.7. Following PCR amplification, samples were heat denatured to form single-stranded DNA and electrophoresed under different conditions. Gels were silver-stained to visualise the different conformers (BioRad). The percentage of polyacrylamide, ratio of acrylamide:bis, amount of glycerol, and temperature at which electrophoresis was carried out were altered to optimise the resolution of conformers and obtain reproducibility. We used a heater-cooler system to control the temperature (Bettatech, UK)

To ensure that the SSCP conditions were able to detect a point mutation, positive controls were used which were known to contain a single point mutation or polymorphism. A single positive control for exons 2 - 10 were kindly supplied by Dr Philippe Froguel. In addition, the $Gly^{299} \rightarrow Arg$ missense mutation in exon 8 of the glucokinase gene (Stoffel et al 1992b) was used.

We initially developed conditions to screen exon 8, as we had two positive controls. We then applied these conditions with minor modifications to the other exons. Gestational diabetic subjects, normoglycaemic subjects and positive controls were run.

5.4 RESULTS.

The $Gly^{299} \rightarrow Arg$ missense mutation in exon 8 had been detected within our department in Pedigrees BX and AX using SSCP (Stoffel et al 1992b). We

initially tried to replicate these methods for this exon, using sample from members of Pedigree BX who possessed the exon 8 mutation as a positive control. We failed to reproduce the results using the same conditions and therefore had to develop our own conditions for SSCP and expand them to include all the exons from 2-10.

By altering the SSCP conditions, with regard to the polyacrylamide gel and electrophoresis parameters, the conformation of the single-stranded DNA may be altered; these conditions are optimised to detect the mutations.

Figures 5.4.1-3 show 3 SSCP gels for exon 8 of the glucokinase gene. The band patterns for each gel were produced by different conditions. This shows how altering the conditions can affect the conformation of the ssDNA and produce changes in the electrophoretic mobility of a sample.

Figure 5.4.1. 6% polyacrylamide without glycerol, run at 25°C. The positive controls from Pedigree BX have the same band pattern as the normoglycaemic controls. The low and high bands are both singlets. The French positive controls have low and high doublet bands, and can therefore be distinguished from the other samples.

Figure 5.4.2. 6% polyacrylamide with 5% glycerol, run at 25°C. These are the same conditions previously used by my colleagues to screen for the $Gly^{299} \rightarrow Arg$

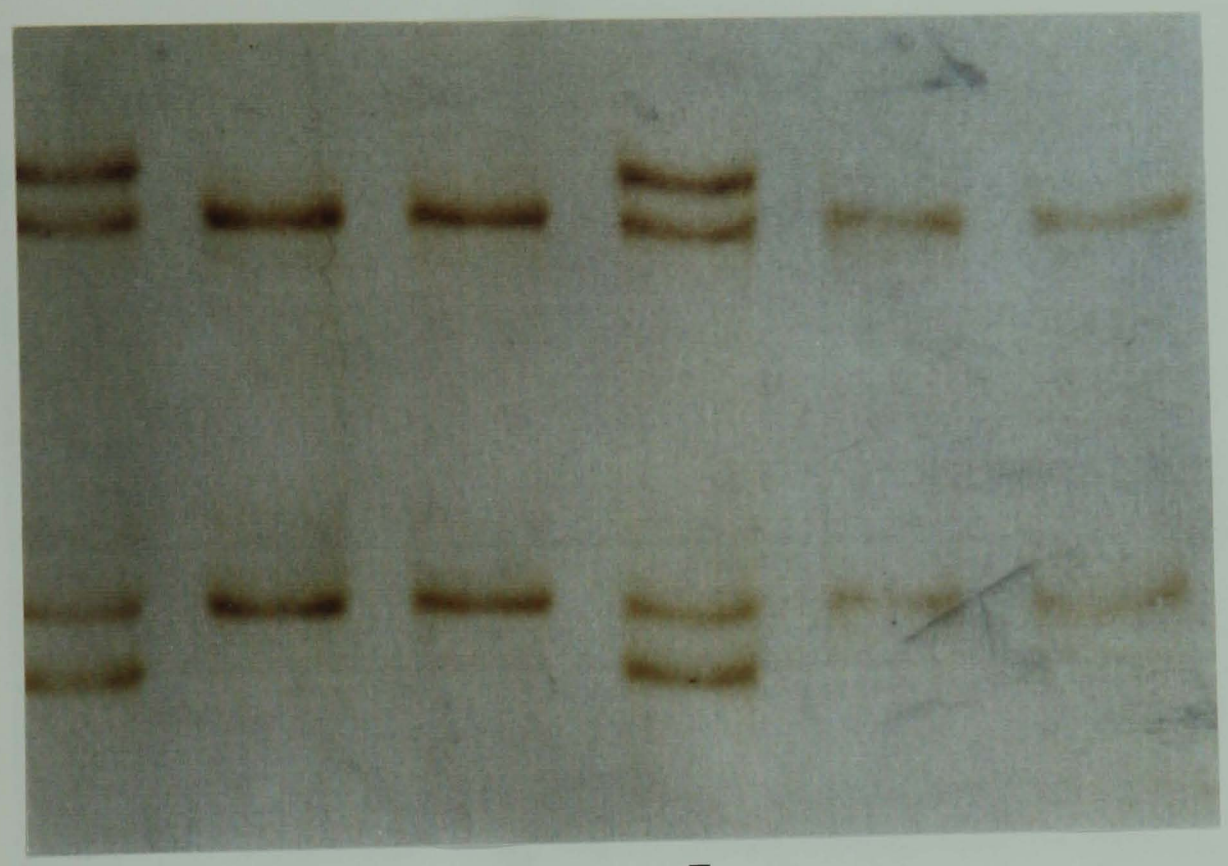
missense mutation in members of Pedigree BX (Stoffel et al 1992b). The positive samples from this Pedigree are indistinguishable from the normoglycaemic controls. The French positive control possesses a low doublet, and unlike the previous conditions no high doublet.

Figure 5.4.3. 10% polyacryalmide with 5% glycerol, run at 25°C. With these conditions each of the positive controls and the normoglycaemic control subjects could be distinguished. The positive sample from Pedigree BX possess a high doublet. The French positive controls have the low and high doublet previously seen in Figure 5.4.1.

The positive controls were used to establish which gel conditions were suitable for mutation screening of each respective exon. They were run together under various SSCP gel conditions along with normoglycaemic controls. For each exon, we chose the gel conditions that enabled us to distinguish the positive control samples from the normoglycaemic samples (Table 5.4). We were able to identify all of the positive control samples for exons 2-10.

During method development an abnormal conformer in exon 8 of the glucokinase gene was detected in two samples (Figure 5.4.4). The positive control was not wholly reproducible at this time. After confirmation on 3 separate SSCP gels from the same product, the samples were sequenced. They were found to possess normal "wild-type" sequences (sequencing up to 16 clones). Reamplification

Figure 5.4.1 6% polyacrylamide gel without glycerol, run at 25°C.

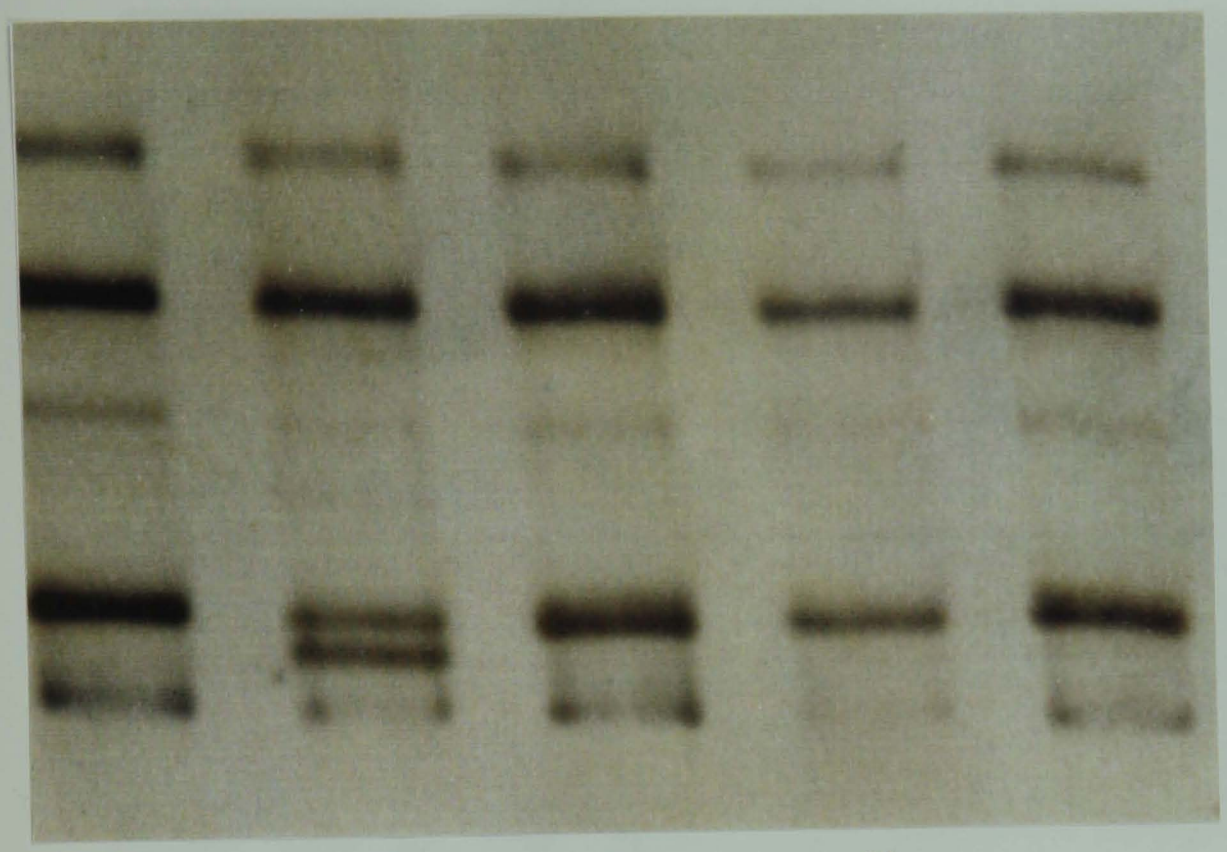


F French positive control

B Pedigree BX positive control

N Normoglycaemic control

Figure 5.4.2 6% polyacrylamide gel with 5% glycerol, run at 25°C.

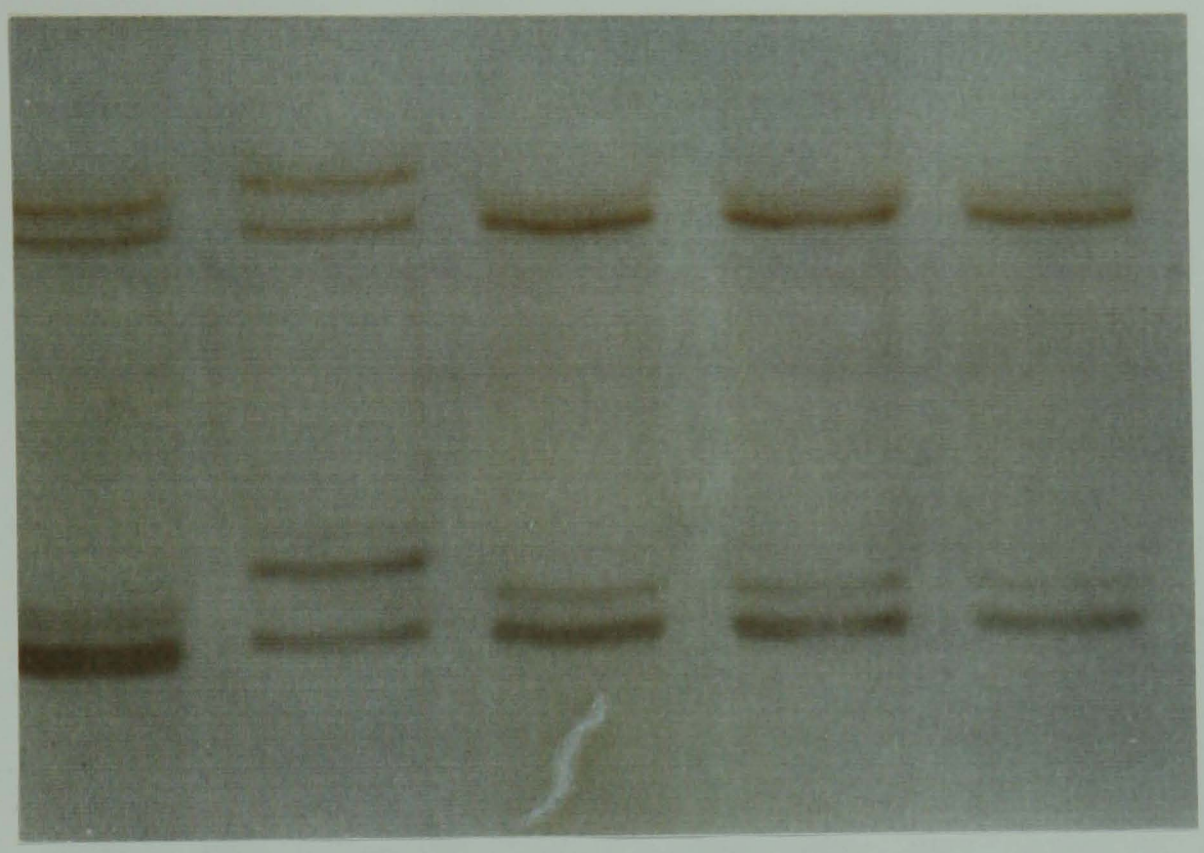


F French positive control

B Pedigree BX positive control

N Normoglycaemic control

Figure 5.4.3 10% polyacrylamide gel with 5% glycerol, run at 25°C.

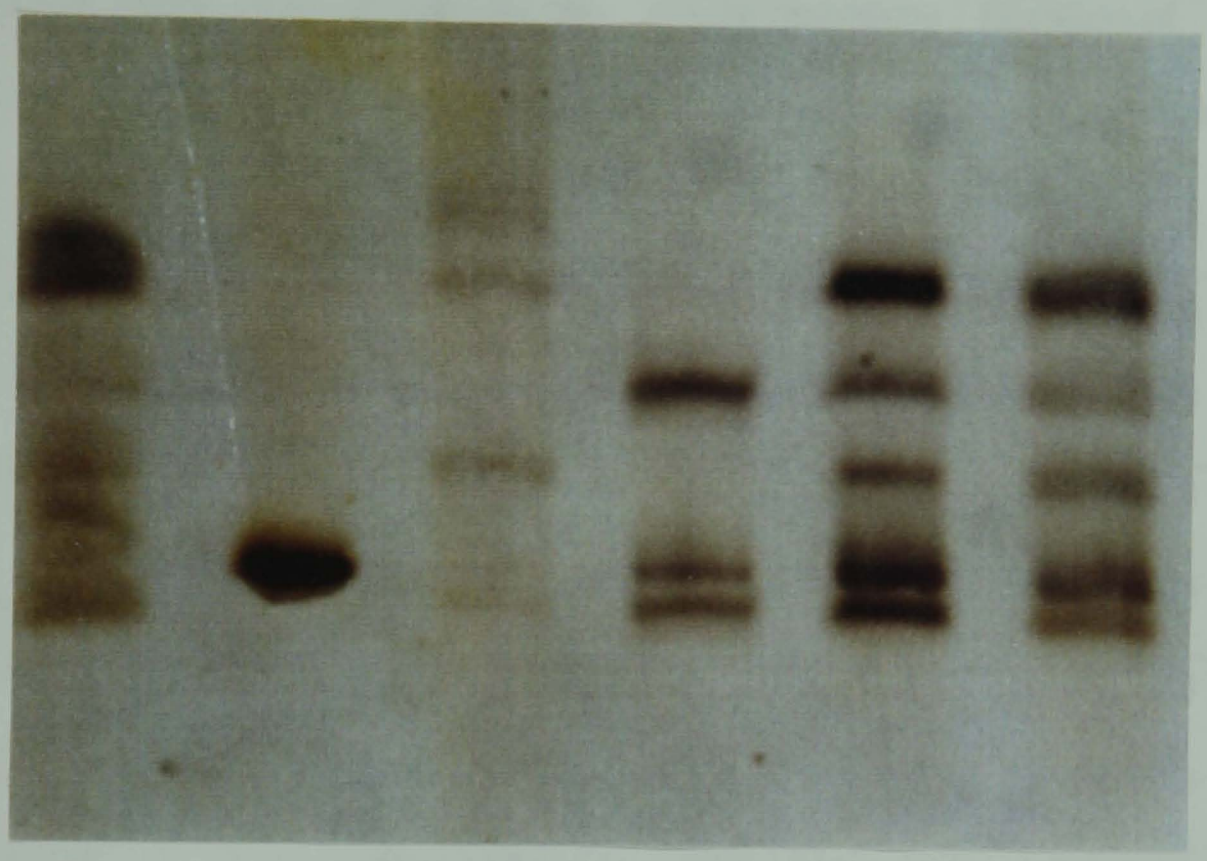


F French positive control

B Pedigree BX positive control

N Normoglycaemic control

Figure 5.4.4 6% polyacrylamide gel with 5% glycerol, run at 25°C.



B Pedigree BX positive control

GA Gestational diabetic subjects with abnormal band patterns

N Normoglycaemic control

G gestational diabetic subject

The abnormal conformer was found to have been produced by normal 'wild-type' sequence.

Table 5.4 SSCP conditions used for mutation screening of exons 2-10 of the glucokinase gene.

GCK exons	Polyacrylamide gel conditions	Temperature gel is run at.	Duration of electrophoresis at 350V
2	10% without glycerol 10% without glycerol	4 °C 25 °C	18 hours 18 hours
3	10 % without glycerol 10% without glycerol	4 °C **20 °C**	18 hours 18 hours
4	10% without glycerol 10% without glycerol	4 °C 25 °C	18 hours 18 hours
5 & 6	10% without glycerol 10% **with 5% glycerol**	4 °C 25 °C	**6 hours** **6 hours**
7	10% without glycerol 10% without glycerol	4 °C **18 °C**	18 hours 18 hours
8	10% without glycerol 10% **with 5% glycerol**	4 °C 25 °C	18 hours 18 hours
9	10% without glycerol 10% without glycerol	4 °C 25 °C	**6 hours** **6 hours**
10	10% without glycerol 10% without glycerol	4 °C 25 °C	18 hours 18 hours

from genomic DNA and SSCP did not continue to show abnormal conformers.

5.5 DISCUSSION.

A method to screen for mutations in exons 2-10 of the glucokinase gene was developed using the technique of SSCP analysis and silver-staining thus negating the requirement for radioactivity. Although SSCP had been used by members of our department in a laboratory 3 miles away, when the conditions were transferred we were unable to detect the $Gly^{299} \rightarrow Arg$ missense mutation in exon 8 of the glucokinase gene. This shows that optimum conditions may vary between laboratories and technicians and has been reported by other workers (Elbein et al, 1993a).

The final protocol was found to be accurate and reproducible and could detect all of the positive controls we possessed. It was established that for each exon the conditions selected were able to detect single base changes in DNA sequence resulting in a polymorphism or mutation. There still remains the possibility that some conformers may be missed. Developmental work showed the importance of having DNA samples with known mutations and "wild-type" samples to act as quality controls to ensure that each gel resolves different conformers and that no problems have occurred with the equipment and parameters during electrophoresis. If appropriate controls are unavailable for the particular exon or gene being studied, it is preferable to run other controls of the same length with

the understanding that the conditions may not be optimal for the exons of interest. General conditions may by used to detect base changes and these can be further optimised to gain clearer gels for photography and publication.

SSCP can detect between 80 - 95% of all single base changes with appropriate conditions (Orita et al 1989). Sheffield et al have investigated the sensitivity of SSCP in the detection of single-base substitutions (Sheffield et al 1990). They found that the resolution of single-stranded DNA is improved with a fragment size <200bp, with an optimum size of approximately 150bp. They found a reduction in the sensitivity as the size of the fragment increases. Results suggested that there was a minimum size limit to detection, this is probably due to the constraints placed on the ability of a small fragment to form stable secondary structures. The ability of a fragment to form stable conformers is also affected by the base sequence surrounding a base change. No significant differences were found for the detection of transitions (81%) or transversions (76%). The lowest detection rate for any class of mutations was for G to T transitions (57%), detected at a significantly lower rate than for all other mutations (79%).

The explanation of the abnormal conformers in exon 8 seen during method development is uncertain, but may be due to errors in PCR amplification with Taq polymerase. Once SSCP conditions had been optimised, no abnormal conformers in the samples were detected in exon 8. This finding shows that false positives may occur because of the formation of more than one conformer for a

particular sequence and has been supported by other workers (Elbein et al, 1993a).

Robust SSCP conditions were developed which had been proven to detect all of the known glucokinase mutations in our possession. This meant that we could progress to the screening of subjects for mutations in exons 2-10 of the glucokinase gene, and be confident of detecting the majority of single-base changes.

CHAPTER 6

MUTATIONS IN THE GLUCOKINASE GENE CONTRIBUTE TO THE PATHOGENESIS OF GESTATIONAL DIABETES

6.1 INTRODUCTION.

We observed that subjects with glucokinase mutations often present with gestational diabetes. 7 of 11 parous women from two pedigrees (AX and BX) with a missense mutation in exon 8 of the glucokinase gene ($Gly^{299} \rightarrow Arg$) were initially diagnosed with gestational diabetes (Hattersley et al 1992b; Stoffel et al 1992). Gestational diabetes has a similar clinical phenotype to subjects with MODY possessing a glucokinase mutation, in that they have an early age of onset, mild hyperglycaemia leading to symptomatic diabetes in later life, and tend to have a strong family history of diabetes (Froguel et al 1993; Page et al 1995). The frequency of glucokinase mutations in subjects with gestational diabetes is not known.

6.2 AIMS.

To investigate the prevalence of gestational diabetes caused by mutations in the glucokinase gene by screening subjects using the technique of single-stranded conformational polymorphism (SSCP) analysis.

6.3 METHODS.

6.3.1 Subjects.

50 unrelated Caucasian subjects from the Oxford region who had been diagnosed as having Gestational diabetes (GDM) were studied. Gestational diabetes was diagnosed on the basis of 2 abnormal oral glucose tolerance test (OGTT) values during pregnancy (28-34 weeks) and with hyperglycaemia (>5.5 mmol/l) on follow up (mean 10 years) (MD Gillmer 1983). Subjects known to be members of pedigrees AX and BX were excluded from the study. The clinical characteristics of the subjects are shown in Table 6.3.

6.3.2 Mutation screening.

Blood samples from the 50 subjects were taken into EDTA and DNA extracted from whole blood using the standard phenol/chloroform method. Mutations in exons 2-10 of the glucokinase gene have been found to be a cause of hyperglycaemia. Mutation screening of these exons was carried out using the technique of single-stranded conformational polymorphism (SSCP) analysis (Orita et al 1989). PCR amplification of exons 2-10 and SSCP analysis was performed as described in Chapter 2; 2.8.1-2.8.7 and Chapter 6. Generally, 2μl of PCR product was run for 18 hours at 350V on 10% polyacrylamide gels under two conditions; with 5% glycerol at 25°C and without glycerol at 4°C.

Table 6.3 Clinical characteristics of the gestational diabetic subjects.

	GDM
Number of subjects	50
Fasting Plasma Glucose mean +/- sd, mmol/l.	6.8 +/- 1.6
Age mean +/- sd, years.	37.0 +/-0.3
BMI mean +/- sd, kg/m^2	29.0 +/- 3.4
Known family history of diabetes	70%

Amplified DNA from a normoglycaemic subject, and for each exon a subject known to possess a glucokinase mutation were run as negative and positive controls. Gels were visualised by silver staining (BioRad).

6.3.3 Sequencing and RFLP analysis.

Exons from subjects shown to have an abnormal band mobility were directly sequenced after isolating single-stranded DNA using biotinylated PCR primers and Dynalbeads (Dynal UK) (Chapter 2; 2.9-2.10.10). The previously characterised missense mutation ($Gly^{299} \rightarrow Arg$) was confirmed by a specific PCR generated RFLP using the restriction endonuclease Hha1 (Stoffel et al 1992b; Chapter 2, 2.7.4).

When a mutation was found, other members of the subject's family were obtained and tested for glucose tolerance, and the presence of the mutation by use of SSCP and the specific RFLP. Paternity was established using DNA finger-printing and highly polymorphic markers (ICI Diagnostics).

6.4 RESULTS.

After screening 50 gestational diabetic subjects, abnormal band patterns were detected in 3 exons (Table 6.4). The results of the normoglycaemic subjects suggested that the abnormal band patterns seen for exons 3 and 9 are due to

Table 6.4 Location of abnormal conformers and the number of GDM subjects and Normals found to possess them.

		GDM	Normals
Exon 3		8/50	10/50
Exon 8		3/50	0/100
Exon 9	a	10/50	12/52
	b	2/50	0/52

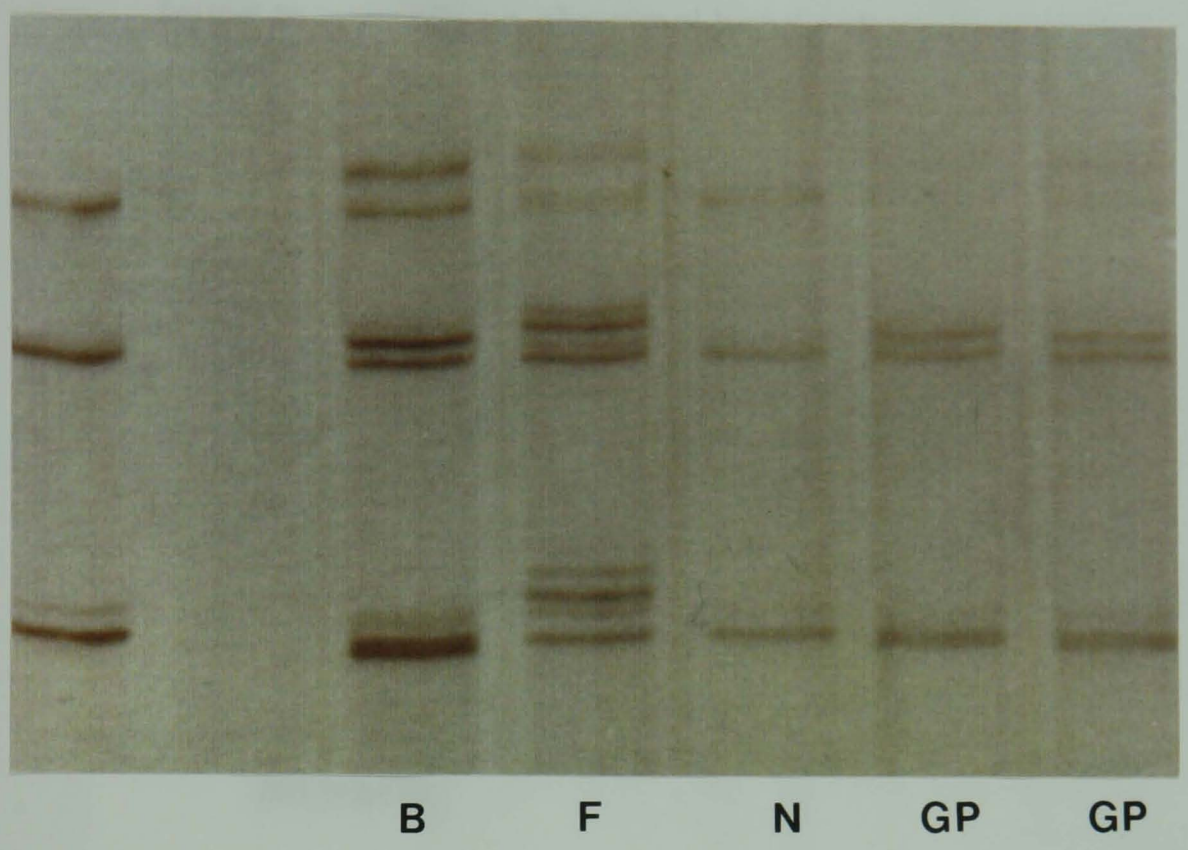
Two abnormal conformers were identified in exon 9, here designated a and b.

polymorphisms. Direct sequencing of exons 3 and 9 has failed to identify a base change resulting in a mutation or polymorphism.

In exon 8 we had already described a mutation in this exon in two pedigrees (AX and BX). 3 unrelated subjects had a similar abnormal band pattern mobility to members of Pedigrees AX and BX who possessed a glucokinase mutation (Figure 6.4.1). This particular mutation can be detected as a restriction fragment length polymorphism (RFLP), as the presence of this mutation creates a cutting site for the restriction enzyme Hha1. Direct sequencing and the use of the RFLP confirmed that these 3 subjects had the $Gly^{299} \rightarrow$ Arg missense mutation found in Pedigrees AX and BX (Figures 6.4.2 and 6.4.3). This mutation in exon 8 has not been found to occur in 100 non-diabetic individuals screened using SSCP and the RFLP.

We further investigated the families of these 3 gestational diabetic subjects by sampling available family members and studying family records. In two subjects one parent and at least one other relative had diabetes compatible with dominant inheritance. The mutation segregated with diabetes and glucose intolerance. In the third subject, neither parent had hyperglycaemia or diabetes. Subsequent testing showed that there was non-paternity, so the family could not be fully studied.

Figure 6.4.1 SSCP gel for Exon 8.



B Pedigree BX positive control

F French positive control

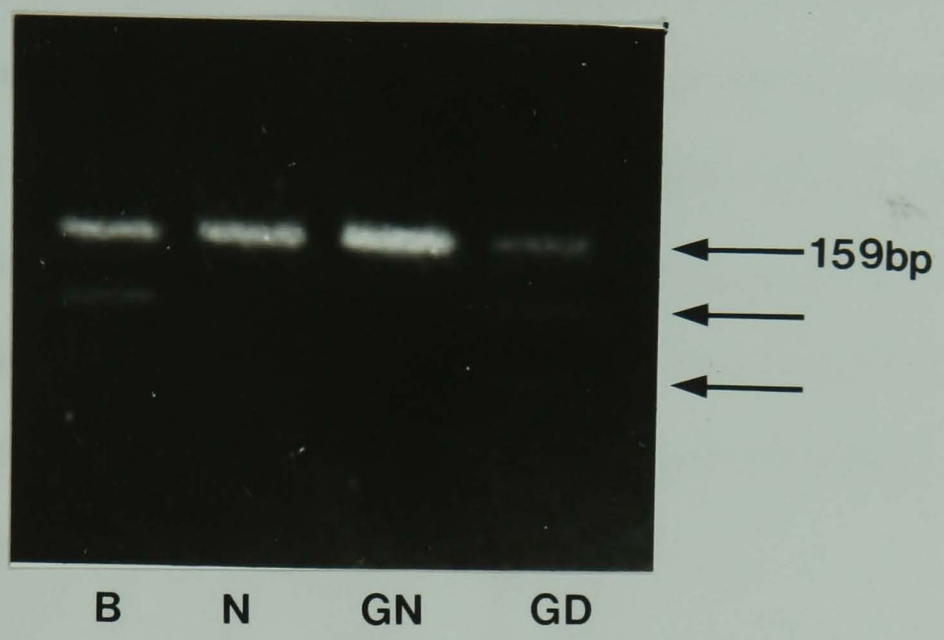
N Normoglycaemic negative control

GP Gestational Diabetic subject with abnormal SSCP band pattern

The gel shows a gestational diabetic subject possessing an abnormal band pattern for exon 8.

Figure 6.4.2 Sequence data for one of the gestational diabetic subjects found to possess an abnormal band pattern in exon 8.

Figure 6.4.3 RFLP for the exon 8 mutation $Gly^{299} \rightarrow Arg$.



B Pedigree BX positive control

N Normoglycaemic negative control

GN gestational diabetic with normal SSCP band pattern

GD gestational diabetic with abnormal SSCP band pattern

The RFLP confirmed that the gestational diabetic subject possessed the previously described $Gly^{299} \rightarrow Arg$ missense mutation.

6.5 DISCUSSION.

This study shows that mutations in the glucokinase gene can contribute to the pathogenesis of gestational diabetes. On screening 50 subjects, we found that 3 unrelated subjects possessed a missense mutation ($Gly^{299} \rightarrow Arg$) in exon 8 of the glucokinase gene. This same mutation has been previously described in two pedigrees (AX and BX) (Stoffel et al 1992b). This mutation results in glucose intolerance: 27 of the 28 individuals possessing the mutation have a fasting plasma glucose greater than 6 mmol/l

A model of human β-cell glucokinase has been constructed based on the related yeast hexokinase B (Gidh-Jain et al 1993; R StCharles et al 1994). These two enzymes share 31% identity and all known catalytic and glucose-binding residues are conserved in these enzymes. This model enables the assessment of the effect of mutations on the molecular mechanism of the glucokinase enzyme. The Gly^{299} residue is highly conserved between species and is located in the region of the molecule leading to the cleft of the active site (Figure 6.5). It has been predicted that the charge alteration associated with the glycine to arginine mutation alters the conformation and stability of the enzyme.

Kinetic enzyme studies have been carried out in E. coli to discover how this gene mutation affects the function of the enzyme molecule (Gidh-Jain et al

Figure 6.5 Model of the glucokinase molecule.

A ribbon drawing of the α-carbon backbone of glucokinase, based on the yeast hexokinase B structure in the open conformation. The position of the $Gly^{299} \rightarrow Arg$ is indicated (Gidh-Jain et al 1993).

1993). The results of this are given in Table 6.5. The Km for glucose showed no significant difference; Km for ATP remains the same. The missense mutation reduces the Vmax of the glucokinase molecule to approximately 1% of the native form of the enzyme, thus its glucose phosphorylating activity is greatly reduced. This decrease in function effects insulin secretion from the β-cell. The $Gly^{299}\rightarrow$ Arg mutation is heterozygous, one of the patient's alleles is normal and therefore only 50% of the glucokinase enzyme is abnormal. This is seen in other glucokinase mutations, a homozygous glucokinase mutation would have severe effects and would be incompatible with life.

Meglasson and Matchinsky (1990) postulated that a decrease in glucokinase enzyme activity may result in a reduced insulin secretion in relation to blood glucose levels. They suggested that a mere 15% reduction in glucokinase activity might shift the set point for glucose induced insulin secretion from 5 to 6 mM. It is uncertain what the overall effect will be if 50% of the enzyme has greatly reduced activity. It is interesting that affected members from the MODY pedigree BX have an approximately 50% decrease in β-cell function (Hattersley et al 1992b; Page et al 1995). This suggests an important regulatory role for glucokinase, and expression of the enzyme is unable to compensate for the mutation's inactivity. The mutant glucokinase enzyme probably does not interfere with the function of the normal enzyme, particularly as it is monomeric. The defect in glucose metabolism is probably due to a gene-dose effect; there is less normal-functioning enzyme present. No changes in DNA sequence were identified by

Table 6.5 Results of kinetic enzyme studies on glucokinase carried out in E. coli

	V max, U/mg	Km Glucose, mM	Km ATP, mM
Native liver GCK isozyme	98 +/- 9	6.8 +/- 1.3	0.23 +/- 0.19
Native β-cell GCK isozyme.	100 +/- 8	8.0 +/- 2.0	0.15 +/- 0.18
$Gly^{299} \rightarrow$ Arg mutation	approx. 0.32 approx. 1% native	3.1 +/- 2.1	0.15 +/- 0.10

No significant difference in the Km for glucose. Km for ATP remains the same. Reduction in the Vmax of the mutant glucokinase molecule to approximately 1% of the native form (Gidh-Jain et al 1993).

direct sequencing for the abnormal conformers found in exons 3 and 9. There are a number of reasons for this occurring. The abnormal conformer may be due to a PCR artefact created by Taq polymerase error, the exon may not have been amplified with high fidelity. This seems unlikely as the SSCP appearance was consistent and reproducible. Alternatively the abnormal conformer may actually be another form of that for the normal allele. There may be problems with direct sequencing of certain abnormal conformers. It has been suggested that primer annealing error may occur resulting in preferential binding to the normal allele thus producing a signal for this alone (personal communication Dr Choudury, Institute of Molecular Medicine, Oxford).

Dr Mark McCarthy has endeavoured to sequence the abnormal conformers he found in exon 3 on the Applied Biosystems (ABI) automatic sequencer using the Sequenase kit. He found that this was not a robust method and had problems with obtaining sequence data and reproducibility. He then used the alternative of Taq sequencing with the ABI. This is reported to be less sensitive at detecting heterozygous mutants. To date, he has not been able to identify any abnormal DNA sequences in the abnormal conformers he detected (personal communication).

Other studies have now been published which have assessed the contribution of mutations in the glucokinase gene to gestational diabetes. All of these utilised SSCP to screen the subjects. Mutations have been found in Caucasians (Zouali

et al 1993; Stoffel et al 1993) and an Hispanic (Stoffel et al 1993), but not in African-Americans (Chiu et al 1993). The results suggest that 5-10% of gestational diabetes in Caucasians and Hispanics may be due to mutations in the glucokinase gene (Zouali et al 1993; Stoffel et al 1993; and this Chapter 6).

The detection of the missense mutation in exon 8 of the glucokinase gene has clinical significance. All 24 subjects from Pedigrees AX and BX with this glucokinase mutation had mild hyperglycaemia which was diet or tablet controlled. Most were non-obese and would have a good prognosis, with little tendency to macrovascular complications (Hattersley et al 1992b; Page et al 1995). Subjects with a glucokinase mutation tend to need insulin during pregnancy (9/11), although they are otherwise diet or tablet controlled. 50% of the subjects' children are at-risk of possessing this mutation. In view of the good prognosis, it is doubtful whether screening them is justified, although it may provide "good" news for those with the mutation because of the relatively "mild" phenotype. It would be more feasible if rapid and accurate screening was possible. Individuals with the same mutation have been classified as having MODY, classical Type 2 diabetes or gestational diabetes depending on when they have been diagnosed.

CHAPTER 7

FOUR PEDIGREES WITH THE SAME GLUCOKINASE MUTATION, POSSIBLY DUE TO A FOUNDER - EFFECT

7.1 INTRODUCTION.

Work carried out in Oxford has focused on the role of the glucokinase gene in Type 2 diabetes. Our results have identified five probands from the Oxford district who possess the same missense mutation in exon 8 of the glucokinase gene. One of these individuals presented with maturity onset diabetes of the young (MODY), one presented with Type 2 diabetes, and three presented with gestational diabetes (GDM). The mutation we found was a missense mutation $Gly^{299} \rightarrow Arg$ in exon 8; it is the only glucokinase mutation to be found in five probands. We suspected the role of a founder-effect in this area as a cause of this high prevalence.

7.2 AIM.

To assess whether the high prevalence of the $Gly^{299} \rightarrow Arg$ glucokinase mutation in the Oxford district is due to a founder-effect.

7.3 SUBJECTS.

7.3.1 Screening of MODY pedigrees.

Six large multi-generation pedigrees were investigated using the microsatellite marker GCK1 and GCK2 (Hattersley AT 1992b; Chapter 3). One of these

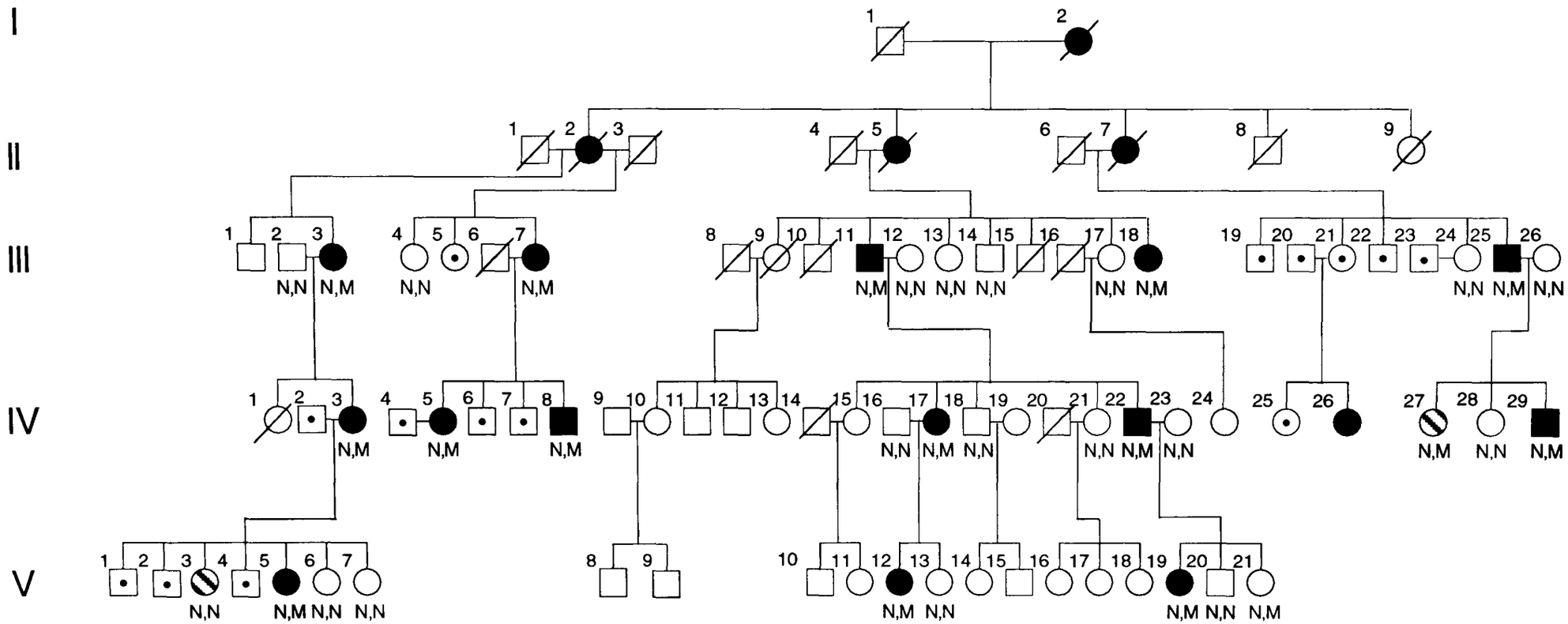
pedigrees, Pedigree BX (Figure 7.3.1) was found to have diabetes mellitus linked to the glucokinase gene. Mutation screening, using the technique of single-stranded conformational polymorphism (SSCP) analysis was carried out. Subjects within Pedigree BX possessing the z+4 allele, which segregated with diabetes, produced an abnormal band pattern in exon 8, and sequencing identified the mutation to be a missense mutation resulting in $Gly^{299} \rightarrow Arg$. This mutation was found to be identifiable as an RFLP as it created a cutting site for the restriction endonuclease Hha1 (Stoffel 1992b).

7.3.2 Screening of Type 2 diabetic patients.

Colleagues screened 50 Type 2 diabetic subjects with diagnosis >40 years old for mutations in exons 7 and 8 using SSCP and RFLPs (Stoffel et al 1992b). Of these, one subject was found to possess the missense mutation in exon 8. 100 non-diabetic subjects screened do not possess this mutation, further confirming that this mutation is the cause of diabetes.

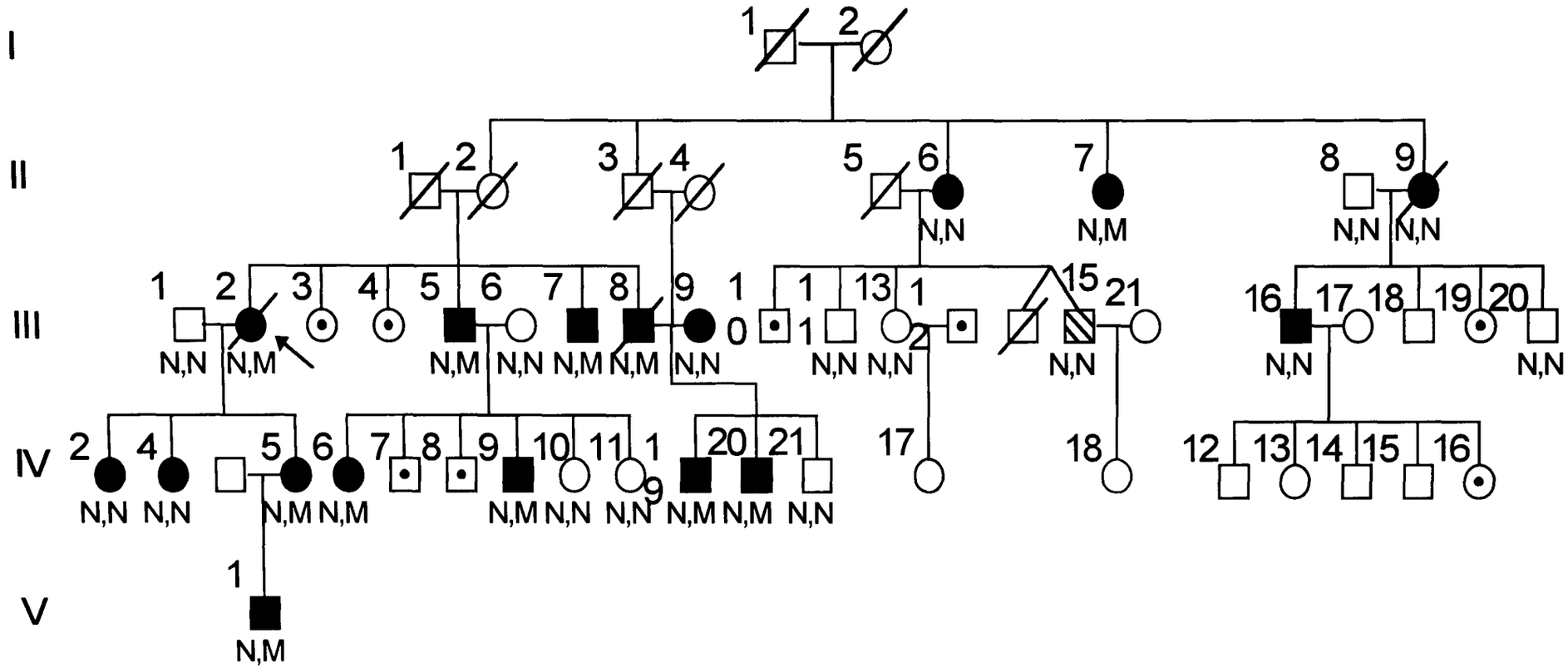
The Type 2 diabetic subject with the mutation is a member of a large multi-generation pedigree, Pedigree AX (Figure 7.3.2). Pedigree extension and mutation screening has increased the number of informative members. All 10 members with the mutation have Type 2 diabetes, one of whom was diagnosed at the age of 19 years. 6 other diabetic members do not possess the mutation (Stoffel et al 1992b), this demonstrates the genetic heterogeneity of diabetes

Figure 7.3.1 Pedigree BX.



N=normal allele; M= mutant allele

Figure 7.3.2 Pedigree AX.



mellitus within a single pedigree.

7.3.3 Screening of gestational diabetic subjects.

We embarked on a study to screen for glucokinase mutations in 50 Caucasian subjects who had had gestational diabetes (Chapter 6). 3 of these gestational diabetic subjects were found to have this same mutation in exon 8. Known members of Pedigrees AX and BX had been excluded from this study.

7.4 METHODS.

There are a number of potential reasons for this high prevalence of a single mutation. It may be that this is a common mutation which occurs spontaneously, or that the probands shared a common ancestor. To differentiate the cause of the high prevalence of this one mutation we carried out:

7.4.1 Pedigree extension.

Family records were studied back to the mid to late 19th Century.

7.4.2 Haplotype construction.

The subjects we had found to possess the mutation were investigated using the

microsatellite polymorphisms GCK1 and GCK2, and haplotypes were constructed.

7.4.3 Screening of at-risk subjects from the same geographical area.

The at-risk subjects we investigated had a fasting plasma glucose in the range 5.5-7.8 mmol/l, and had been entered into the Fasting Hyperglycaemia Study. 108 subjects from Oxford were screened for this mutation in exon 8 of the glucokinase gene using the specific RFLP.

7.5 RESULTS.

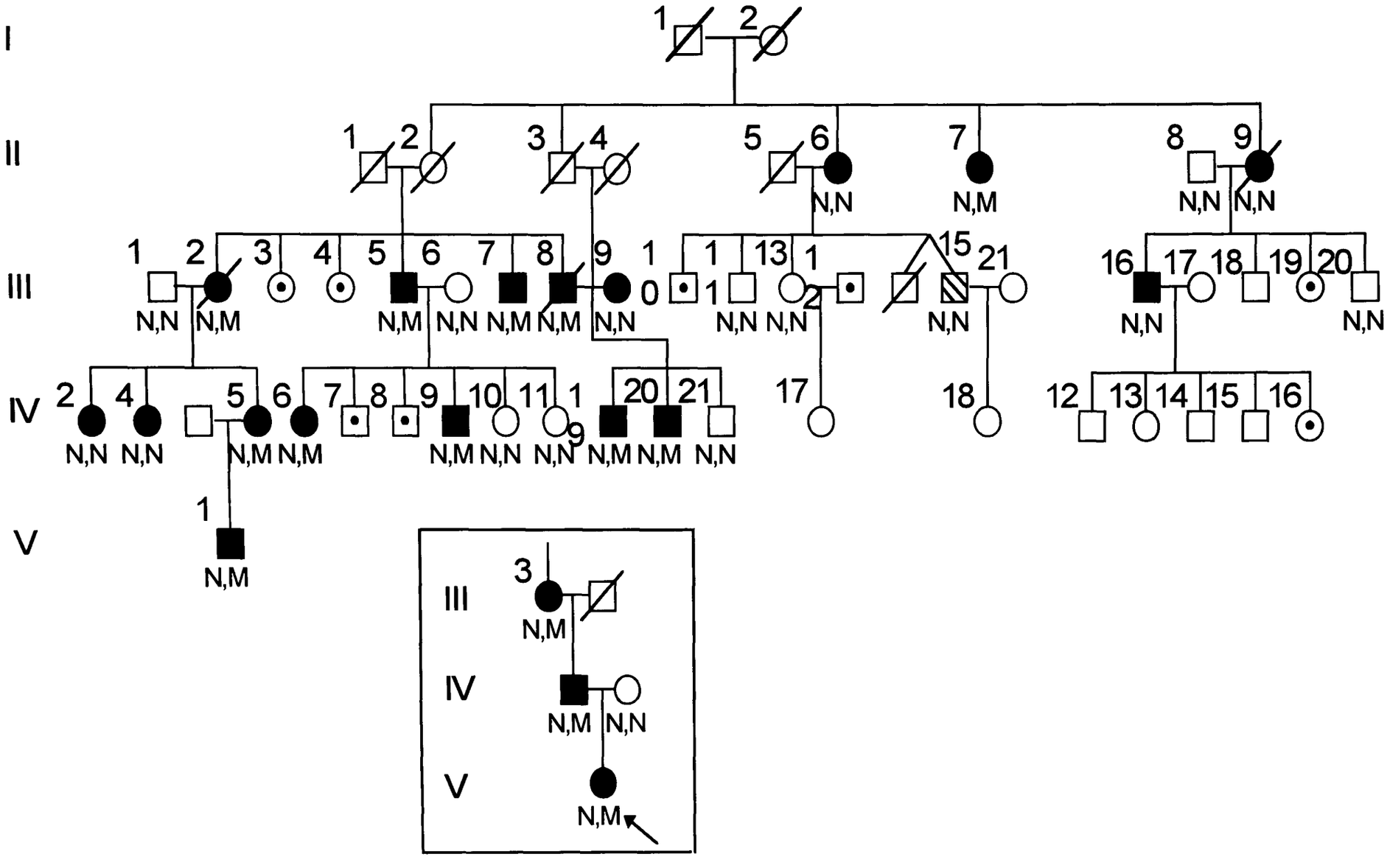
7.5.1 Pedigree extension.

After Pedigree extension we found that one of the gestational diabetic subjects is a member of a previously unknown branch of Pedigree AX (Figure 7.5.1). The gestational proband is related via her paternal grandmother who had been unavailable for sampling on all previous studies into this Pedigree.

7.5.2 Haplotype construction.

Using the microsatellite markers GCK 1 and 2, many of the subjects with the mutation were found to have the haplotype z+4/2 (Table 7.5.2). In those in whom it was not possible to assign a haplotype, the alleles did contain both z+4 and 2,

Figure 7.5.1 Pedigree AX showing the relationship with the gestational diabetic proband.



N - normal allele; **M** - mutant allele; arrow indicates gestational diabetic proband.

Table 7.5.2. Allele scores for the microsatellite markers GCK1 and GCK 2 for subjects with the $Gly^{299} \rightarrow$ Arg mutation. From these, haplotypes were constructed when at least one of the polymorphisms was homozygous.

Subject	GCK1	GCK2	Haplotypes
Pedigree AX			
II-7	Z,Z+4	2,2	Z,2 **Z+4,2**
IV-5	Z,Z+4	2,2	Z,2 **Z+4,2**
IV-19	Z,Z+4	2,2	Z,2 **Z+4,2**
IV-20	Z,Z+4	2,2	Z,2 **Z+4,2**
V-1	Z+2,Z+4	2,	Z+2,2 **Z+4,2**
Pedigree BX			
IV-5	Z,Z+4	2,2	Z,2 **Z+4,2**
IV-9	Z,Z+4	1,2	
IV-10	Z,Z+4	1,2	
IV-19	Z,Z+4	1,2	
IV-33	Z+4,Z+4	2,3	**z+4,2** z+4,3
V-5	Z+4,Z+4	2,2	**Z+4,2**
Gestationals			
KD (AX)	Z,Z+4	2,2	Z,2 **Z+4,2**
RW (AX)	Z+4,Z+4	2,2	**Z+4,2**
MB	Z+4,Z+4	2,2	**Z+4,2**
MA	Z+4,Z+4	1,2	z+4,1 **z+4,2**

so the haplotype could have occurred.

7.5.3 Screening of at-risk subjects from the same geographical area.

None of the at-risk subjects were found to possess the mutation.

7.6 DISCUSSION.

Using linkage analysis of a MODY pedigree and mutation screening followed by sequencing of this pedigree, classical Type 2 diabetic subjects and Gestational diabetic subjects we have identified five probands who possess the same missense mutation in exon 8 ($Gly^{299} \rightarrow Arg$) of the glucokinase gene. This is the only glucokinase mutation to be described in five separate probands. All five probands reside in the Oxford district, and this mutation has not been described outside this area.

Pedigree extension revealed that one of the three gestational diabetic subjects was a member of a previously unknown branch of Pedigree AX. The gestational proband is related to this family via her paternal grandmother. This Pedigree had been classified as being a classical Type 2 pedigree (O'Rahilly et al 1988 & 1989) which had been found to have a glucokinase mutation. The finding of this new branch of the Pedigree would support the suggestion that the diabetes in the Pedigree members with the glucokinase mutation was early-onset and hence not

a different phenotype from Pedigree BX. KD had been diagnosed as having gestational diabetes at the age of 24 years. In addition, we tested the son of Pedigree member IV-5. He possesses the mutation and at the age of 19 years has a fasting plasma glucose of 7.1 mmol/l. This means that two members of Pedigree AX had been diagnosed with hyperglycaemia before 25 years of age. This Pedigree could now be reclassified as being one with MODY by the criteria proposed by Froguel (Froguel et al 1992); autosomal inheritance and 2 members diagnosed before the age of 25 years.

Construction of haplotypes for those subjects with the GCK mutation found that they all have the z+4/2 haplotype. This suggests that no recombination has taken place in recent evolutionary history. The observed frequency for this haplotype in British Caucasian non-diabetic controls is 17.6% (Chapter 3) The presence of this mutation in these four pedigrees is therefore likely to be due to a common ancestor. The odds of the same haplotype occurring by chance alone if the four probands are unrelated is 1/183 $(0.176)^3$ ie chance of second proband = 0.176; third = $(0.176)^2$, fourth = $(0.176)^3$ which is 0.0054.

We investigated subjects from the Fasting Hyperglycaemia Study who reside in the Oxford area to discover whether this mutation was present in this at-risk group. This mutation was not present in 108 subjects screened using the specific RFLP. Despite this result, the prevalence of other glucokinase mutations may be increased in at-risk populations from the same area as that of the probands.

Using various molecular biological approaches, we have identified a missense mutation in the glucokinase gene which is unique to the Oxford area. Individuals with the same mutation have been classified as MODY, Type 2 diabetes or gestational diabetes depending on when they have been diagnosed. Their molecular diagnosis is that of Glucokinase-deficient glucose intolerance. Pedigree extension and the construction of haplotypes suggests that the observed high prevalence for this mutation is due to a founder-effect.

CHAPTER 8

SCREENING OF SUBJECTS WITH TYPE 2 DIABETES, MODY, AND GESTATIONAL DIABETES FOR THE MITOCHONDRIAL TRANSFER RNA ($tRNA^{Leu(UUR)}$) MUTATION AT POSITION 3243bp.

8.1 INTRODUCTION.

Type 2 diabetes may be associated with defects in mitochondrial DNA (mtDNA) (Ballinger et al 1992). A point mutation (A→G) at position 3243bp within the gene encoding the transfer RNA for leucine ($tRNA^{Leu(UUR)}$) has been found in some subjects who have maternally inherited Type 2 diabetes and/or deafness (van den Ouweland et al 1992; Reardon et al 1992; Remes et al 1993; Oka et al 1993; Kadowaki et al 1993 & 1994). Diabetic subjects with the mutation may present with gestational diabetes (Reardon et al 1992; Alcolado et al 1994). It has been estimated that approximately 1-3% of Type 2 diabetic subjects with a family history of diabetes possess this mutation (Vionnet et al 1994; Katagiri et al 1994; Kadowaki et al 1994). We assessed the prevalence of this mutation in UK Caucasians. We screened 500 random Type 2 diabetic subjects, 748 Type 2 diabetic subjects with a family history of diabetes, 50 subjects who previously had gestational diabetes and 5 MODY pedigrees, 2 of which appeared to show maternal inheritance in the major part of the pedigrees.

8.2 AIMS.

To investigate the contribution of the mitochondrial transfer $tRNA^{Leu(UUR)}$ mutation at position 3243bp to Type 2 diabetes, MODY and gestational diabetes in UK Caucasian subjects.

8.3 SUBJECTS.

500 random subjects with Type 2 diabetes, 748 Type 2 diabetes and a family history of diabetes, 50 subjects who had been diagnosed as having gestational diabetes and subjects from 5 MODY pedigrees were studied.

The 500 random subjects with Type 2 diabetes were from the UK Prospective Diabetes Study. At presentation they were aged 25-65 years, with a fasting plasma glucose >6 mmol/l, on two occasions (UKPDS VIII 1991). A separate cohort of 748 Type 2 diabetic subjects with a family history of diabetes were from the UK Prospective Diabetes Study and had a first degree relative with diabetes. The subjects who had been diagnosed with gestational diabetes (GDM) were identified by MD Gillmer and had previously been investigated for mutations in the glucokinase gene (Chapter 6). The MODY subjects were from five pedigrees (Chapter 3;Figure 3.3.1), two of which (M and EA) appeared to show maternal inheritance in a major part of the pedigree. Linkage to the glucokinase gene had been excluded in these 5 MODY pedigrees (Chapter 3).

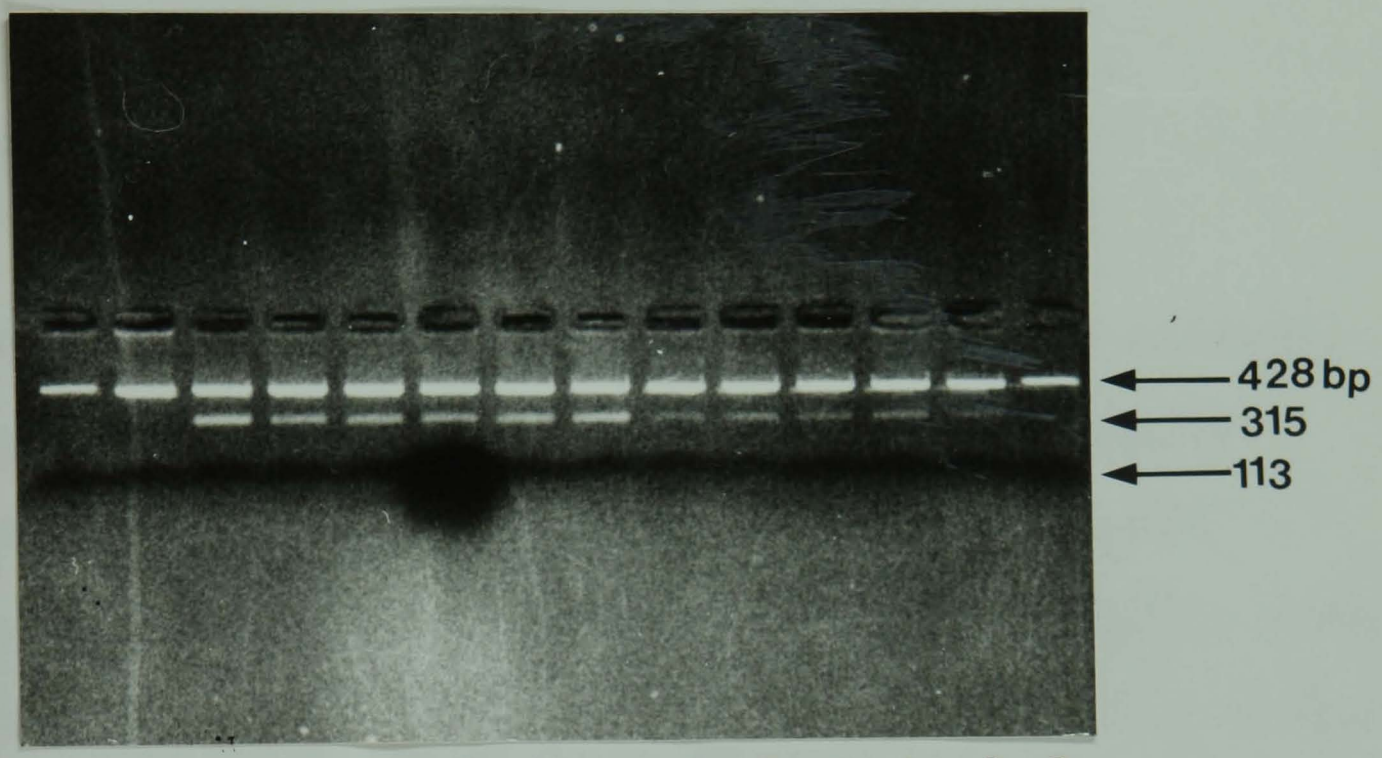
8.4 METHODS.

To screen for the mutation in the mitochondrial transfer RNA, mitochondrial DNA was extracted from leukocytes using the Nucleon method. DNA was amplified by the polymerase chain reaction (PCR) using the primers previously described (van

den Ouweland et al 1992; Chapter 2, 2.7.5) under the following conditions: 1μg DNA, 100μg of each dNTP, 10 mmol/l Tris-HCl (pH3.8), 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, and 1.5 Units of Taq DNA Polymerase; initial denaturing at 96°C for 4 minutes, then 30 cycles of denaturing at 93°C for 30 seconds, annealing at 52°C for 30 seconds, extension 72°C for 40 seconds, and a final extension of 72°C for 10 minutes. PCR product was visualised on a 1.5% agarose gel with ethidium bromide staining. 10μl of the PCR product was then digested with 20U of Apa1 and incubated at 37°C for 2 hours, followed by electrophoresis on a 3% agarose gel stained with ethidium bromide. Amplified mtDNA from normal and a known mutant (provided by Prof A Harding) were digested and run on each gel as negative and positive controls respectively.

To assess the sensitivity of our procedures we screened a series of mutant mtDNA diluted with normal mtDNA of the same concentration (98% mutant, 80%, 70%, 60%, 50%, 40%, 30%, 20% & 10%, and subsequently 10%, 9%, 8%, 7%, 6% 5%, 4% 3%, 2% & 1%). To compliment this, we also blindly screened 3 subjects known to have less than 5% of mutant mtDNA in their blood (provided by P Smith, Birmingham). Using the dilution series we were able to detect every sample down to 4% mutant DNA diluted with normal DNA (Figure 8.4). The mutation could sometimes be detected at 2%-3%, but this was not invariable. We were also able to detect the mutation in all 3 subjects known to possess less than 5% of mutant mtDNA.

Figure 8.4 Dilution series to assess the sensitivity of the RFLP and ethidium bromide staining to detect the $tRNA^{Leu(UUR)}$ mutation at position 3243bp.



Mutant mtDNA was diluted with normal mtDNA of the same concentration.

ß-cell function (assessed as %ß) and insulin sensitivity (assessed %S) were estimated by Homeostasis Model Assessment (HOMA) (Matthews et al 1985) on the subjects possessing the $tRNA^{Leu(UUR)}$ mutation at 3243bp. It should be noted that HOMA has imprecision of 20-25%.

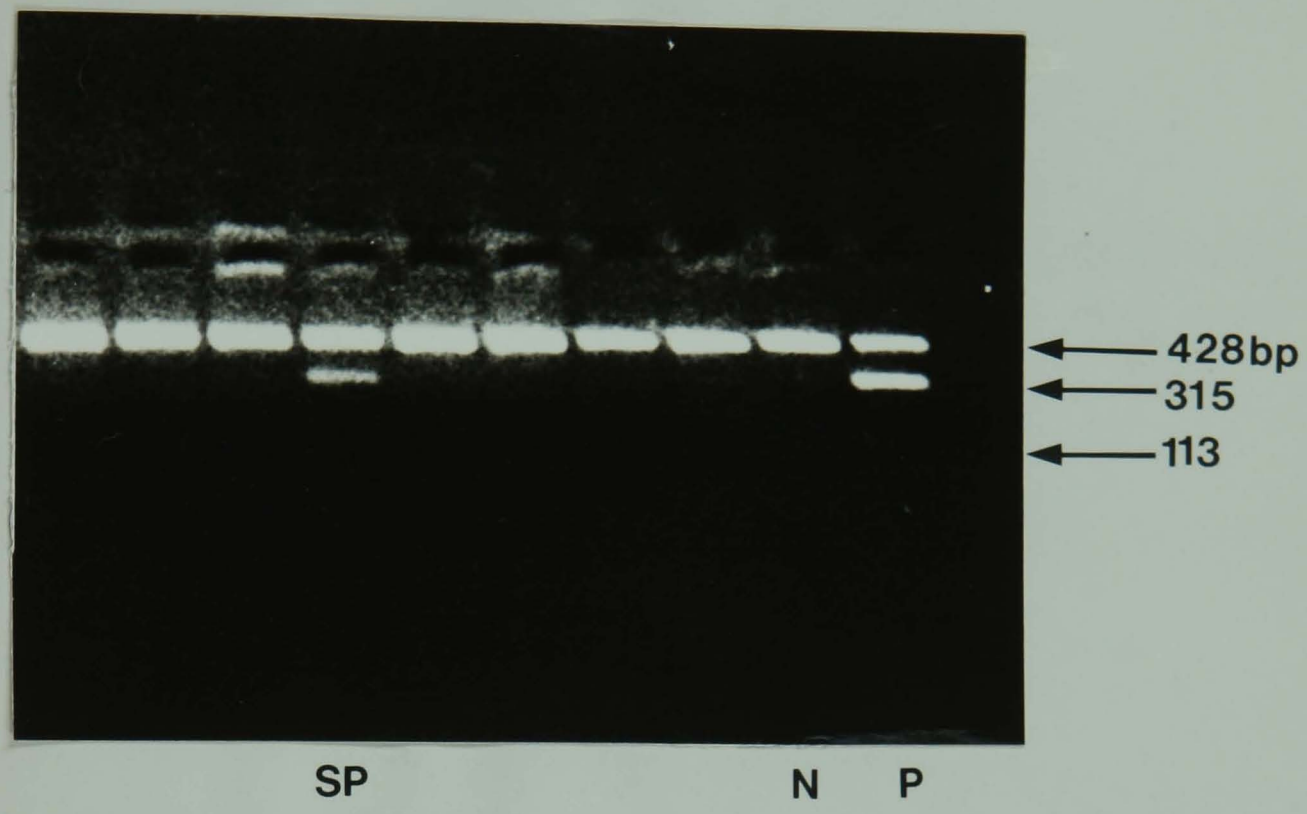
8.5 RESULTS.

We did not detect the $tRNA^{Leu(UUR)}$ mutation in any of the random subjects with Type 2 diabetes mellitus, those who had been diagnosed with gestational diabetes, or members of the MODY pedigrees. We detected the $tRNA^{Leu(UUR)}$ mutation in 2 of the 748 Type 2 diabetic subjects with a family history of diabetes (Figure 8.5.1) Their pedigree trees are shown in Figures 8.5.2. Both showed a maternal mode of inheritance of diabetes. Their clinical characteristics are shown in Table 8.5.1 and clinical details showing progression of their disease is shown in Tables 8.5.2 and 8.5.3. Both patients were non-obese and had reduced ß-cell function as assessed by HOMA. Subject 1 had hyperglycaemia (fpg 17.2 mmol/l) and required insulin therapy, whereas Subject 2 had mild hyperglycaemia (fpg 7.2 mmol/l) and obtained normal fasting plasma glucose level on very small amounts of glipizide.

8.6 DISCUSSION.

The aim of this study was to investigate the contribution of the mitochondrial

Figure 8.5.1 Agarose gel showing RFLP analysis for the $tRNA^{Leu(UUR)}$ mutation at position 3243bp.



SP Subject detected with the mutation

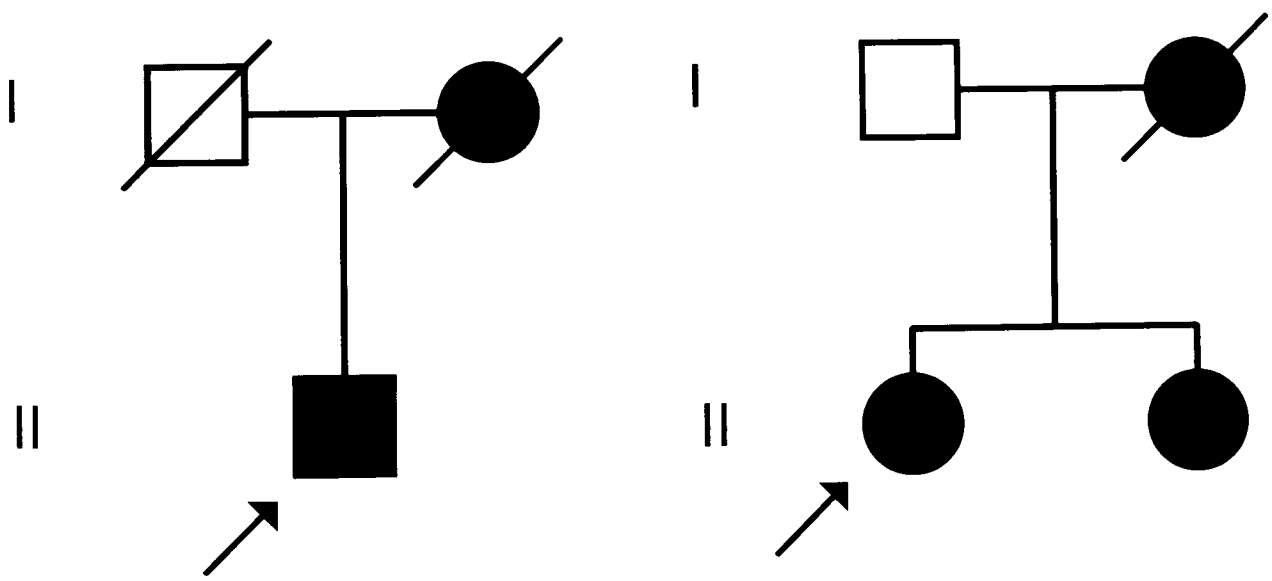
N normoglycaemic negative control

P positive control

The other samples shown are from diabetic subjects with a family history of diabetes.

The mitochondrial DNA mutation was detected in 2 of the diabetic subjects with a family history of diabetes.

Figure 8.5.2 Pedigree trees of the two subjects possessing the $tRNA^{Leu(UUR)}$ mutation at 3243bp.



Subject 1

Subject 2

Table 8.5.1 Clinical characteristics of the two subjects found to possess the $tRNA^{Leu(UUR)}$ mutation at position 3243bp.

	SUBJECT 1	SUBJECT 2
Sex	Male	Female
Age at diagnosis	38 years	36 years
Fasting plasma glucose, mmol/l	17.2	7.2
Fasting plasma insulin, mU/l	10.3	6.7
BMI, kg/m^2	17.2	22.1
%B, HOMA*	13.2%	51.6%
%S, HOMA*	26.4%	51.4%
Deafness	Reported	None reported
Current treatment	Insulin therapy	Diet
Family history of diabetes	Mother, NIDDM	Mother, IDDM Sister, NIDDM

*Homeostasis model assessment (HOMA) using program supplied by JC Levy

Table 8.5.2 Clinical data showing progression of diabetes in Subject 1 possessing the $tRNA^{Leu(UUR)}$ mutation at position 3243bp.

Subject 1						
Time	Weight, Kg	Therapy	fpg, mmol/l	fpi, mU/l	HOMA* %B	HOMA* %S
at diagnosis	52	Diet	16.7	10.3	13	26
3 months	52	Diet	17.2	missing	N/A	N/A
1 year	59	23 U/day insulin	6.1	15.0	N/A	N/A
2 years	60	27 U/day insulin	5.4	12.3	N/A	N/A
3 years	63	34 U/day insulin	8.7	19.0	N/A	N/A
4 years	59	20 U/day insulin	8.9	17.0	N/A	N/A
5 years	58	27 U/day insulin	9.9	19.5	N/A	N/A
6 years	59	27 U/day insulin	11.3	12.1	N/A	N/A
7 years	59	29 U/day insulin	8.9	16.3	N/A	N/A
8 years	60	33 U/day insulin	12.9	10.8	N/A	N/A
9 years	60	32 U/day insulin	6.4	missing	N/A	N/A

*Homeostasis model assessment (HOMA) using program supplied by JC Levy

Table 8.5.3 Clinical data showing progression of diabetes in Subject 2 possessing the $tRNA^{Leu(UUR)}$ mutation at position 3243bp.

Subject 2						
Time	Weight, Kg	Therapy	fpg, mmol/l	fpi, mU/l	HOMA* %B	HOMA* %S
at diagnosis	56	Diet	7.2	6.7	52%	51%
3 months	54	Diet	6.4	5.9	59%	60%
1 year	49	Glipizide 2.5 mg	4.5	4.1	95%	97%
2 year	52	Glipizide 2.5 mg	5.3	5.1	79%	74%
3 year	54	Diet	7.3	17.9	99%	20%

*Homeostasis model assessment (HOMA) using program supplied by JC Levy

$tRNA^{Leu(UUR)}$ mutation to Type 2 diabetes, MODY and gestational diabetes in UK Caucasian populations. We failed to detect this particular mutation in the 500 random Type 2 diabetic subjects, members of five MODY pedigrees, and 50 gestational diabetic subjects. 2 of 748 Type 2 diabetic subjects who had a family history of diabetes were found to possess this point mutation. We are confident that our methods are sensitive enough to detect the majority of subjects who possess this mutation as we were able to reliably detect down to 4% mutant DNA in the presence of normal DNA, and in all 3 samples from subjects known to have less than 5% of mutant mtDNA. This mtDNA mutation is unlikely to be a cause of MODY or gestational diabetes in UK Caucasians.

Table 8.6. summarises the results of studies investigating the contribution of this mutation to Type 2 diabetes. The results for the MODY pedigrees and the random Type 2 diabetic subjects agree with previous studies of Vaxillaire, Alcolado, Otobe and t'Hart. Our finding that 0.27% of UK Caucasian Type 2 diabetic subjects with a family history of diabetes possess the mitochondrial $tRNA^{Leu(UUR)}$ mutation at position 3243 bp contrasts with the results of Kadowaki, Vionnet, and Kishimoto who found a prevalence of between 1.9% and 2.8% in their study populations.

There are a number of possible reasons for this difference in prevalence. The results may reflect racial differences between Japanese and UK Caucasians. Although Vionnet studied Caucasians, these subjects were a different population

Table 8.6. Summary of studies investigating the role of the A→G tRNA$^{Leu(UUR)}$ mutation at 3243bp in Type 2 diabetes.

MODY pedigrees		
	Vaxillaire et al (1994)	Saker et al (1995)
No. of positive subjects	0% (0/15)	0% (0/5)

Random Type 2 Pedigrees				
	Alcolado et al (1994)	Otabe et al (1994)	t'Hart et al (1994)	Saker et al (1995)
No. of positive subjects	0% (0/150)	0.9% (5/550)	0.4% (2/473)	0% (0/500)

Subjects with a family history							
	Vionnet et al (1993)	Alcolado et al (1994)	Kadowaki et al (1994)	Katagiri et al (1994)	t'Hart et al (1994)	Kishimoto et al (1995)	Saker et al (1995)
Family history criteria	at least 2 first degree relatives with Type 2 diabetes	at least one affected sibling with Type 2 diabetes	first degree relative with diabetes	mother with Type 2 diabetes	Type 2 diabetes and deafness in at least 2 generations	first degree relative with Type 2 diabetes	first degree relative with diabetes
No. of positive subjects	1.9% (5/267)	1.5% (1/68)	2% (2/100)	1.4% (4/294)	10.7% (3/28)	2.8% (6/214)	0.27% (2/748)

(French rather than UK) and had different ascertainment criteria. Our group of UKPDS subjects were selected if they had a known family history in one first degree relative, whereas the French subjects had at least 2 first degree relatives with diabetes. Our protocol used ethidium bromide staining rather than radioactivity as a detection method. In theory this is less sensitive, but we could reliably detect down to 4% mutant mtDNA.

Alcolado et al and t'Hart et al found a high prevalence of the mutation in their Caucasian subjects with a family history of diabetes. This may be explained by the small sample numbers and the ascertainment criteria. The study of t'Hart et al is particularly important as it shows that when stringent selection criteria are applied, the prevalence of the mutation can be as high as 10%. It also strengthens the evidence for the association of the $tRNA^{Leu(UUR)}$ mutation at 3243bp with the subtype of maternally inherited Type 2 diabetes and deafness (MIDD). 't Hart et al concluded that the previously reported prevalence of the $tRNA^{Leu(UUR)}$ mutation is an over-estimation of its contribution to Type 2 diabetes. Our results are in agreement with this, and suggest that this mutation is not a common contributor to Type 2 diabetes in the UK Caucasian population and is supported by other workers (Alcolado et al 1994).

The clinical data for the two subjects who are positive for this mitochondrial mutation are shown in Table 8.5.1. They both had an early age of diagnosis (M 38 years; F 36 years), and were non-obese. The male was reported to be deaf

and had BMI 17.2 kg/m^2, and had the "more severe" diabetes, with a fasting plasma glucose of 17.2mmol/l, 13.2% normal ß-cell function and required insulin therapy of 35 Units per day, appropriate for a normal weight diabetic (Holman et al 1985). The female was not reported to be deaf and had "milder" disease, with BMI 22.1 kg/m^2, fpg 7.2 mmol/l, 51.6% ß-cell function, and on diet therapy had a fasting plasma glucose of 6.4-7.3 mmol/l, and on glipizide (only 2.5mg/day) fasting plasma glucose of 4.5-5.3 mmol/l. Over the 3 or 9 years follow-up available there was no apparent progression of severity of their disease (Tables 8.5.2 and 8.5.3). Reviewing the literature, diabetic subjects found to possess the $tRNA^{Leu(UUR)}$ mutation show heterogeneity in the clinical phenotypes they display. Type 2 diabetic individuals with the mutation tend to have an earlier age of onset of diabetes, lower BMI, and an earlier requirement for insulin therapy (Awata et al 1993; Kishimoto 1994; Kadowaki 1993 & 1994).

Mutations in mitochondrial DNA provides an example of the heterogeneity of Type 2 diabetes. Defects in enzymes within the mitochondrial apparatus involved in oxidative metabolism, maintenance of glucose homeostasis, or "house-keeping" may contribute to Type 2 diabetes. Diabetes has been found to occur in subjects with deletions and duplications associated with Kearns-Sayre syndrome (KSS) and chronic progressive external opthalmoplegia (CPEO) (Poulton et al 1994). Defects in enzymes coded for by mtDNA may explain the maternal pattern of inheritance exhibited within some Type 2 diabetic pedigrees, as mitochondrial DNA is solely inherited from the mother (Alcolado et al 1995). Further studies

may show more common genetic defects affecting mitochondrial function.

CHAPTER 9

THE GLUCAGON RECEPTOR GENE REGION AND SUSCEPTIBILITY TO TYPE 2 DIABETES

9.1 INTRODUCTION.

Glucagon plays an important role in the control of hepatic glucose production and is involved in the regulation of insulin secretion. Glucagon action is mediated through its binding to a specific receptor which belongs to the superfamily of G protein-coupled transmembrane receptors. Mutations in the glucagon receptor gene located on chromosome 17q25 may therefore contribute to Type 2 diabetes. SSCP mutation screening, and direct sequencing identified a heterozygous mutation in exon 2, resulting in GGT^{40} to AGT ($Gly^{40} \rightarrow Ser$) (Hager et al 1995). This mutation was found in 5% of French familial Type 2 diabetic patients and 0.9% of controls. Receptor binding studies revealed that the presence of this mutation results in a 67% reduction in affinity for the binding of glucagon compared to the "wild-type". This could play a role in the development of Type 2 diabetes as hyperglucagonaemia increases gluconeogenesis and contributes to hepatic insulin resistance (Baron et al 1987; Raskin et al 1978).

Although an association was demonstrated between the GCG-R gene mutation and Type 2 diabetes, genetic linkage was only found when individuals in families with impaired glucose tolerance (IGT) were included (Hager et al 1995). This mutation removes a cutting site for the restriction endonuclease BstEII, so can therefore be detected as an RFLP. In an independent case-control study of Sardinian subjects, they failed to show a statistically significant association. No clinical data were available for those individuals who possessed the mutation. A

rare phenotypic subgroup of Type 2 diabetes can therefore not be excluded.

The results of Hager et al need replicating in an independent data set. Full clinical details are required, to distinguish whether those subjects possessing the mutation are phenotypically different from classical Type 2 diabetic subjects.

9.2 AIMS.

To attempt to replicate the results of the association of the GCG-R gene mutation with Type 2 diabetes in UK Caucasian subjects. To examine the phenotypes, to determine whether those with the mutation exhibited features of classical Type 2 diabetes. To investigate whether this mutation was present in subjects who have an increased risk of developing diabetes by screening individuals who had previously had gestational diabetes, and individuals with fasting hyperglycaemia, but without diabetes as defined by WHO criteria.

9.3 SUBJECTS.

The subjects investigated were as follows:

a. 274 Caucasian Type 2 diabetic subjects from the Oxford centre of the UKPDS (UKPDS VIII 1991).

b. 100 individuals who had previously been diagnosed as having gestational

diabetes.

c. 69 individuals with fasting hyperglycaemia (>5.5, <7.8 mmol/l) on two separate occasions who had been recruited as part of the Oxford centre of the Fasting Hyperglycaemia Study (FHS). None of these had overt diabetes, and none were receiving oral hypoglycaemic agents at the time of recruitment.

d. 5 MODY pedigrees (Chapter 3).

e. 17 nuclear pedigrees who were not linked to the glucokinase gene (Cook et al 1992). Affected subjects had Type 2 diabetes or impaired glucose tolerance, as shown by an intra-venous glucose tolerance test.

f. 146 non-diabetic controls were recruited as part of the UKPDS.

9.4 METHODS.

Exon 2 was amplified using the primers described by Hager et al, and shown in Chapter 2; 2.7.6. Genomic DNA was amplified in a total volume of 25μl. PCR conditions were: initial denaturation at 94°C for 5 minutes; 32 cycles of 94°C for 90 seconds, 55°C for 1 minutes, and 72°C for 30 seconds with a final extension at 72°C for 10 minutes. PCR product was checked on a 1.5% agarose gel. 7μl of the PCR product was digested with 5U of BstEII at 60°C for 2 hours. The samples were then loaded onto a 3% agarose, 1% NuSieve gel with ethidium bromide staining and electrophoresed. Digestion products were visualised using UV-light.

9.4.1 Statistical Analysis

The Fisher's right-tailed exact test was used to assess whether there was significant differences in the frequency of the mutation in the diabetic groups and non-diabetic controls.

Differences between the clinical characteristics of UKPDS Type 2 diabetic subjects with and without the mutation were analysed using the students t-test.

9.5 RESULTS.

Mutation of the GCG-R gene was present in 7/274 Oxford UKPDS subjects and in 0/146 UKPDS non-diabetic controls. The results showed an association of the mutation with Type 2 diabetes (diabetics vs non-diabetic controls, 2.6% vs 0%; p=0.049). The clinical characteristics of the patients from the UKPDS Type 2 diabetes study in Table 9.5 .1 show no differences in phenotype between those with and without the GCG-R mutation. 2 patients with gestational diabetes were found to possess the mutation (GDMs vs non-diabetic controls, 2% vs 0%). Data was available for 50 of the gestational diabetic subjects who at present have mild hyperglycaemia (Table 9.5.2). There were no differences between the subject with or the 49 subjects without the mutation with respect to the characteristics of pregnancy (including duration of gestation, number of previous miscarriages and the weight of the child), or clinical characteristics at follow-up (including body

Table 9.5.1 Clinical characteristics of patients in the UKPDS Type 2 diabetes study.

	Type 2 Diabetic subjects negative for the GCG-R gene mutation (n=267).	Type 2 Diabetic subjects positive for the GCG-R gene mutation (n=7)
Age, years.	52.3 (+/-8.6)	52.3 (+/-8.4)
Body Mass Index, Kg/m^2.	29.2 (+/-5.7)	32.3 (+/-6.4)
Fasting plasma glucose, mmol/l.	12.6 (+/-4.2)	13.0 (+/-2.5)
Fasting plasma insulin, mU/l.	14.2 (8.2 to 24.5)	21.1 (17.8 to 25.1)*
Insulin sensitivity, %S.	21.5 (12.7 to 36.5)	14.4 (11.7 to 17.7)**
β-cell function, %B.	33.6 (15.3 to 73.8)	38.2 (25.8 to 56.4)
Waist/hip ratio	0.92 (+/-0.09)	0.94 (+/-0.10)

Results adjusted for sex, expressed as mean (standard error). Homeostasis model assessment (HOMA) using program supplied by JC Levy. Differences tested using students t-test. Fasting plasma insulin, insulin sensitivity and β-cell function expressed as geometric mean (1 standard error mean) comparisons between groups for these variables were performed on log transformed data.. * p=0.08; ** p=0.06

Table 9.5.2 Characteristics of the gestational diabetic subjects currently with mild hyperglycaemia.

	Gestational diabetic subjects negative for the GCG-R mutation (n=49)	Gestational diabetic subject positive for the GCG-R mutation (n=1)
Family history in first degree relatives	32% yes	yes
Characteristics of pregnancy		GDM diagnosed during third pregnancy
insulin treatment	88% (44/49)	during third pregnancy
No. of weeks gestation	38.7 +/- 2	38.3 +/- 0.5
mean weight of children	7.32 +/- 1.43	8.0
number of children	2.7 +/- 1.3	3
number of miscarriages	0.6 +/- 1.1	0
Characteristics on follow-up		
Age, years	38.6 +/- 6.2	33
No of years since initial diagnosis of GDM	10.6 +/- 5.9	10.0
mean BMI, Kg/m^2	27.8 +/- 6.1	28.5
mean fpg, mmol/l	7.0 +/- 3.7	5.7
mean fpi, mU/l	10.9 (6.19-19.1)	11.7
CIGMA %B	83.5 (55.3-117.5)	117%
CIGMA %S	42.7 (21.5-61.0)	32%

Fasting plasma insulin, insulin sensitivity and β-cell function expressed as geometric mean (1 standard deviation range).

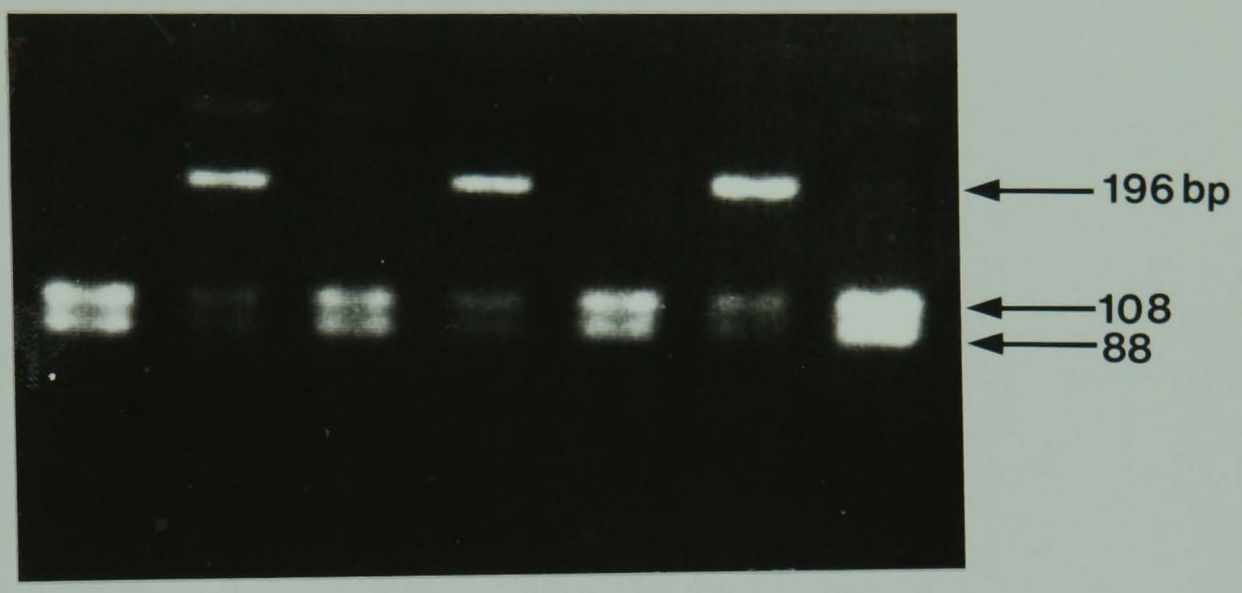
mass index, fasting plasma glucose or fasting plasma insulin). 1 of the subjects with fasting hyperglycaemia was found to possess the mutation (FHS vs non-diabetic controls, 1.4% vs 0%). The frequency of the mutation in these two groups combined is 3/169 (combined vs non-diabetic controls, 1/57 vs 0/149), a similar frequency to that in Type 2 diabetic subjects, but it is not a significant difference from the non-diabetic controls (p=0.153).

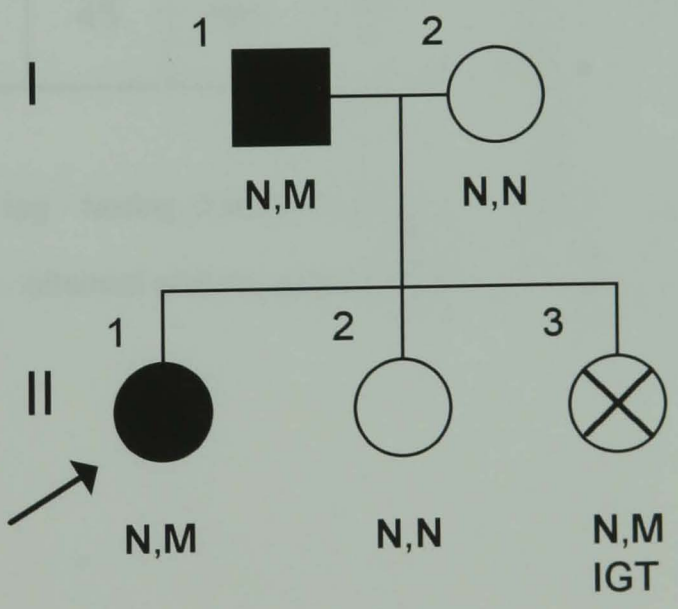
Subjects in one of the nuclear pedigrees were found to possess the GCG-R mutation. The mutation segregated with Type 2 diabetes and IGT and was not found in the non-diabetic mother and sibling. The pedigree tree is shown in Figure 9.5, and the available clinical data is shown in Table 9.5.3. 2 of the 3 subjects with the GCG-R mutation have diabetes. The third subject has normal fasting plasma glucose, but glucose intolerance as assessed with CIGMA. These three subjects all have a low normal ß-cell function. None of the members of the MODY pedigrees were found to have the GCG-R mutation

9.6 DISCUSSION.

We have confirmed the association of a mutation of the GCG-R gene with Type 2 diabetes. This mutation was present in 2.6% of Oxford UKPDS patients and 0% of non-diabetic UKPDS control subjects. Those subjects positive for the mutation showed clinical features of classical Type 2 diabetes. Compared to those subjects without the mutation, there were no significant differences in age at

Figure 9.5 Agarose gel and Pedigree tree of the Nuclear pedigree J10, showing the inheritance of the Gly^{40}→Ser mutation in the glucagon receptor gene.





N - normal allele; M - mutant allele; IGT - impaired glucose tolerance; arrow indicates proband

Table 9.5.3 Clinical data for the three siblings from Nuclear Pedigree J10.

Subject	Sex	Age, years	BMI, Kg/m^2	fpg, mmol/l	CIGMA apg, mmol/l	CIGMA %B	CIGMA %S
II1	Female	36	26.4	N/A	N/A	31.0	122.8
II2	Female	51	N/A	5.36	7.93	126	72.1
II3	Female	45	N/A	5.24	10.58	57.0	70.9

BMI - body mass index; fpg - fasting plasma glucose; CIGMA - continuous infusion of glucose model assessment; apg - attained plasma glucose (CIGMA 1 hour glucose);

diagnosis, body mass index, fasting plasma glucose, or HOMA estimates (Matthews et al 1985) of ß-cell function or insulin sensitivity.

Genetic linkage of the GCG-R gene mutation to Type 2 diabetes had previously been found only when data was combined from individuals with IGT. Hager et al speculated that this mutation may play a role in the early development of some forms of Type 2 diabetes by increasing the susceptibility to IGT. We therefore investigated the prevalence of this mutation in individuals who had been diagnosed with gestational diabetes, and those who had fasting hyperglycaemia. Members of these groups are at an increased risk of developing Type 2 diabetes in later life. The observed frequency of the GCG-R gene mutation in these two groups combined (1.8%) was similar to that found in Type 2 diabetes (2%), but was not significantly different from non-diabetic controls. A signifcant difference would have been consistent with the hypothesis that this mutation increases the susceptibility to IGT which then progresses to Type 2 diabetes. This hypothesis is supported by one of the nuclear pedigrees though. The GCG-R gene mutation was found to segregate with Type 2 diabetes and IGT in Pedigree J10 (Figure 9.5).

Glucagon receptors are found in the liver and β-cells. In the liver, glucagon stimulates glycogenolysis and gluconeogenesis resulting in an increase in plasma glucose concentration (Magnuson et al 1995). Glucagon also stimulates insulin secretion which partially regulates the increased production of glucose from the

liver. In normal controls, glucagon has been shown to contribute to the maintenance of hepatic glucose output (Magnuson et al 1985). In type 2 diabetic subjects, glucagon levels are involved in sustaining more than 50% of the increased basal output of hepatic glucose (Baron et al 1987).

Hager et al conducted functional analysis studies and found that the receptor had reduced affinity for glucagon in the presence of the mutation. These results were for the homozygous form, but only the heterozygous GCG-R mutation has been detected. A mutation could result in an increased turnover of the glucagon-receptor, a direct decrease on the physiological response to glucagon, or another mechanism. Hager et al had preliminary data (unpublished) which suggested that the baby hamster kidney cAMP response to glucagon was lowered. This would support a decrease in the physiological effect of glucagon in the presence of the mutant GCG-R.

The trend to higher fasting plasma insulin and insulin sensitivity in those subjects with the GCG-R mutation was not statistically significant, and probably reflects the increased BMI in these patients, or the relatively small number of subjects involved. As glucagon appears to act mainly through the GCG-R (Kawai et al 1995), a mutation which resulted in weaker glucagon binding and a decrease in cAMP response might be expected to produce a decreased rather than increased physiological effect of glucagon.

Alternatively, if the mutation decreases the stimulatory effect of glucagon on the β-cell, the mutation may result in a decrease in the insulin secretory response. The clinical data did not indicate that those with the mutation had less β-cell function than other Type 2 diabetic subjects.

The data from the Nuclear pedigree shows linkage of diabetes/IGT with the mutation, suggesting a prominent role. On the other hand, the mutation is also found in non-diabetic subjects (Hager et al 1995; Gough et al 1995). It is possible that the Gly^{40}→Ser GCG-R mutation is in linkage disequilibrium with another, as yet unknown, mutation in or near the GCG-R gene. This hypothesis would explain why the described mutation segregates in pedigrees, where it is linked with a pathogenic mutation, but can also be found in the normal population (10/1122 normal subjects Hager et al 1995; 1/279 normal subjects Gough et al 1995), where it has not segregated with the pathogenic mutation.

The positive association without a pathogenic role for the mutation, and linkage disequlibrium could have occurred due to genetic admixture. The group studied here are well-selected, but such problems can occur even in populations previously thought to be homogeneous, ie Nauruans. This explanation may not wholly be correct, as these cohorts of subjects have been used in previous population association studies with negative results. This would suggest that they are well-matched. Unlike this study though, they did not utilise bi-allelic markers which are more sensitive to genetic admixture than CA repeats with multiple

alleles. It would be useful to construct a model of the GCG-R molecule to assess the effect of the mutation on its molecular mechanism. Kinetic studies should also be conducted to discover how this GCG-R mutation affects the function of the GCG-R and the physiological response to glucagon. Measurements of the glucagon concentration in the subjects who possess the mutation could be compared to matched Type 2 diabetic and non-diabetic controls to assess whether there is a compensatory increased response in glucagon secretion.

We have confirmed an association of the glucagon receptor gene mutation ($Gly^{40} \rightarrow Ser$) with Type 2. Despite this, there is insufficient evidence to define a role for this mutation in Type 2 diabetes. This study illustrates the difficulty in establishing the role of a base sequence variation, even if it is known to alter the amino acid sequence. This remains one of the major challenges to defining the molecular genetics of Type 2 diabetes.

CHAPTER 10

DISCUSSION

The work described in this dissertation has studied a number of different subject groups to investigate the molecular genetics of Type 2 diabetes, with particular emphasis on the contribution of glucokinase, a specific mitochondrial DNA mutation, and a mutation in the glucagon receptor gene.

Mutations in the glucokinase gene had been found to be the cause of hyperglycaemia in subjects with MODY, a subgroup of Type 2 diabetes (Froguel et al 1992b; Hattersley et al 1992b). This subgroup had been studied as it is clinically well-defined, and its early age of onset enables the collection of large multi-generation pedigrees for genetic studies. We investigated the inheritance of two polymorphic microsatellite markers at the glucokinase loci in another five British MODY pedigrees. The results of this linkage study revealed that defects in the glucokinase gene are an unusual cause of diabetes in British MODY pedigrees with symptomatic hyperglycaemia. The results further highlighted the heterogeneity of MODY, and further studies have shown that there are at least four genes involved in MODY. In addition to glucokinase, loci near the ADA gene (Bell et al 1991) on chromosome 20, and recently loci on chromosome 12q (MODY3) (Froguel et al 1995; Dronsfield et al 1995), have been to be linked to diabetes. The genes and mutations responsible have yet to be identified. There is at least one other gene contributing to MODY as some pedigrees are not linked

to any of these three loci.

Following identification of glucokinase mutations being shown to be responsible for members of the MODY subgroup of Type 2 diabetes, we completed a population association. No association of the glucokinase locus with Type 2 diabetes in the UK Caucasian population was found. This result suggested that a single major mutation in or near the glucokinase gene is not a major cause of Type 2 diabetes in this population. It doesn't negate a role for a number of minor mutations, or a major role in certain patient populations. Mutation screening has identified over 20 different glucokinase mutations in MODY, but has shown that pathogenic mutations in Type 2 diabetes are rare.

The development of a robust method for SSCP analysis, enabled us to screen for mutations. We showed that mutations in the glucokinase gene can contribute to the pathogenesis of gestational diabetes. Diagnosis based on the identification of this genetic lesion enabled us to predict a good prognosis based on the clinical observations in affected MODY subjects possessing this particular mutation.

Using various molecular biological approaches, five probands had been identified with the same missense mutation in the glucokinase gene. This particular mutation was unique to the Oxford area. Individuals with the same mutation had been classified as MODY, Type 2 diabetes, or gestational diabetes depending on when they had been diagnosed. Their molecular diagnosis is that of glucokinase-

deficient glucose intolerance. Pedigree extension and the construction of haplotypes suggested that the observed high prevalence for this mutation was due to a founder-effect. The data suggest unsuspected glucokinase mutations in extended family members may be more common than is realised.

From these studies, we can conclude that defects in the glucokinase gene are not a major contributor to Type 2 diabetes in UK Caucasians, but may have a prominent role in subgroups of this disease. More studies need to be conducted to determine whether there is a role for defects of the glucokinase promotor region, and any modifying genes. The effect of mutations of hepatic glucokinase on liver function have yet to be fully assessed. If there is reduced hepatic glucokinase activity, it might result in insulin resistance in the liver. Insulin resistance was not found to be increased in subjects with the $Gly^{299} \rightarrow Arg$ mutation (Hattersley et al 1992b; Page et al 1995).

Methods may be developed to rapidly screen for mutations. This will probably be achieved by an automated system. Subjects with a phenotype similar to that of glucokinase-deficient glucose intolerance may then be tested for defects of the glucokinase gene. This molecular genetic approach to diagnosis may then provide information on the prognosis and aid treatment.

Under aerobic conditions, 95% of ATP synthesis in β-cells is supplied by the mitochondrial respiratory chain. Defects in mitochondria may therefore affect

insulin secretion and glucose homeostasis. The $tRNA^{Leu(UUR)}$ mutation at position 3243 bp has been found in a number of pedigrees with diabetes mellitus and/or deafness. We investigated the contribution of this $tRNA^{Leu(UUR)}$ mutation in the UK Caucasian population. We found that 0.27% (2/748) of Type 2 diabetic subjects with a family history of diabetes possess this mutation. This finding contrasts with the prevalence of 1.9-2.8% in other study populations (Vionnet et al 1994; Katagiri et al 1994; Kadowaki et al 1994). Differences in race, or the ascertainment of subjects may be responsible for this. The clinical data of our two positive subjects and a review of the literature shows that diabetic subjects possessing the $tRNA^{Leu(UUR)}$ mutation show heterogeneity in their clinical phenotypes. There is a suggestion that Type 2 diabetic individuals with the mutation tend to have an earlier age of onset of diabetes, lower BMI, and an earlier requirement for insulin therapy.

Further investigations of the relationship between mitochondria and diabetes may continue to utilise RFLP analysis, mutation screening and direct sequencing of the mitochondrial genome. A recent finding is that subjects with Kearns-Sayre syndrome and mtDNA duplications often have diabetes (Poulton et al 1994). This is not the first time that duplications have been reported to be associated with diabetes. The improvement in the DNA polymerases used in PCR will enable amplification of up to 10kb, thus allowing relatively rapid screening of the mitochondrial genome. It may then be possible to establish whether there is a role for mtDNA duplications in Type 2 diabetes. The interaction of nuclear

encoded mitochondrial enzymes including manganese superoxide dismutase, is still to be fully investigated.

We confirmed the association of the heterozygous mutation in the glucagon receptor gene ($Gly^{40} \rightarrow Ser$) with Type 2 diabetes. It is difficult to interpret the putative physiological abnormality and how the defect results in diabetes, unless the receptor defect hinders the glucagon stimulation of ß-cell secretion. This mutation may be in linkage disequilibrium with another mutation in, or near the glucagon-receptor gene, such as the promotor region. The physiology of glucagon would suggest over-expression of the receptor in the liver might lead to hyperglycaemia. It has been suggested that these are false- positive associations, due to genetic admixture between the diabetic and control subjects. This explanation may not be wholly correct as the subjects studied in Chapter 9 have been used in previous population association studies with a negative result (Chapter 4; Zhang et al 1994). Previous studies though, utilised CA repeats with multiple-alleles rather than bi-allelic markers which are much more sensitive to demonstrating genetic admixture. The contribution of this mutation to Type 2 diabetes still needs to be clarified.

Future Studies.

The discovery of further gene defects contributing to Type 2 diabetes will require the identification of suitable subjects to study. Physiological studies will help to

differentiate the phenotypic heterogeneity and define further subgroups suitable for investigation. One approach may be the study of genes responsible for diabetic complications such as cardiovascular disease and the relationship with cholesterol. Family studies are still important, but to overcome the problems involved with obtaining large pedigrees the collection of sib-pairs has been proposed. This strategy also has inherent problems but will still be of great value. There is the possibility of establishing a UK BDA-coordinated collection for these subjects, as has already been successful with Type 1 diabetes and MODY pedigrees.

There has been an advancement in the technology which may be applied to linkage studies. Genome-wide screening using the polymorphic microsatellite markers is now a realistic proposition. This requires no knowledge of the biochemical defect, or the identification of a candidate gene. The polymorphic markers are fluorescently labelled, and many markers can be run on the same gel and subsequently analysed. The equipment used increases the amount of information which can be obtained at a given time and hence the speed. This approach has been successful in Type 1 diabetes (Davies et al 1994). It has recently been applied to Type 2 diabetes with the finding of linkage to chromosome 12q in MODY pedigrees (Froguel et al 1995; Dronsfield et al 1995). Now MODY3 has been isolated to this region, studies will now progress to identify the gene involved and the genetic defect contributing to the disease. The work carried out to determine the genetic lesion near the ADA loci illustrates the

possible problems which will be encountered during this project.

The mutations which have been shown to contribute to Type 2 diabetes to date are only responsible for a small proportion of this polygenic disease. Elucidating the genes responsible for the common polygenic form of Type 2 diabetes remains a difficult challenge. Power calculations suggest that very large data sets will be required to establish the role of a new gene in Type 2 diabetes. This will require collaboration between research groups and a highly mechanised approach to genetic analysis. Success will depend on clinical and physiological studies, animal studies and the application of new molecular genetics techniques.

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ABBREVIATIONS.

ADP	adenosine diphosphate
cAMP	cyclic adenosine monophosphate
ATP	adenosine triphosphate
dATP	deoxyadenosine triphosphate
ddATP	dideoxyadenosine triphosphate
bp	base pair
dCTP	deoxycytidine triphosphate
ddCTP	dideoxycytidine triphosphate
DNA	deoxyribonucleic acid
cDNA	complementary DNA
mtDNA	mitochondrial DNA
ssDNA	single-stranded DNA
EDTA	ethylenediaminetetra-acetic acid
FHS	Fasting Hyperglycaemia Study
GCK	glucokinase

dGTP	deoxyguanosine triphosphate
ddGTP	dideoxyguanosine triphosphate
dH_2O	distilled water
HLA	human leucocyte antigen
kb	kilo base
Km	Michaelis constant
LOD	log_{10} of odds
MODY	maturity-onset diabetes of the young
dNTP	deoxynucleotide triphospate
ddNTP	dideoxynucleotide triphosphate
OD	opical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
RFLP	restriction fragment polymorphism
RNA	ribonucleic acid

tRNA	transfer RNA
RT	room temperature
SDS	sodium dodecyl-sulphate
SSCP	single-stranded conformational polymorphism
TBE	tris-boric EDTA buffer
TE	tris-EDTA buffer
TEMED	N, N, N', N'-tetramethyl-ethylenediamine
Tris	tris(hydroxymethyl) amino methane
TTP	thymidine triphosphate
dTTP	deoxythymidine triphosphate
ddTTP	dideoxythymidine triphosphate
UKPDS	UK Prospective Diabetes Study
UV	ultraviolet

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